Glucocorticoids, 11β-Hydroxysteroid Dehydrogenases and Macrophage Function

James Stewart Gilmour

BSc. Hons

Thesis presented for the degree of

Doctor of Philosophy

University of Edinburgh

August 2002

Molecular Endocrinology,
Molecular Medicine Centre,
Western General Hospital,
University of Edinburgh.

&

Centre for Inflammation Research,
Medical School,
Teviot Place,
University of Edinburgh.

Abstract

Glucocorticoid (GC) therapies are widely used to treat chronic inflammatory diseases. Synthetic GCs can augment the safe clearance of apoptotic, yet potentially histotoxic cells by 2 classes of phagocytes- macrophages (Mϕs) and mesangial cells (MC) - in a dose dependent, glucocorticoid receptor (GR)-mediated fashion *in vitro*. Furthermore, failure to clear apoptotic cells *in vivo* has been implicated in the progression of tissue injury, chronic inflammation and autoimmunity. Recently it has been found that pre-receptor metabolism of GCs by tissue specific 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) can act as an important determinant of GC action by reactivating GCs from inert 11-keto forms. Sufficient levels of unbound 11-keto products circulate and may therefore provide a means to increase intracellular GC concentrations in specific cells. This study characterises 11β-HSD1 expression and activity in rat MC and cells of human and murine Mϕ lineage, and suggests an immunomodulatory role for murine 11β-HSD1 in the course of sterile, acute peritonitis.

Firstly, all phagocytes studied expressed 11β-HSD1 mRNA and the resulting enzyme activity was exclusively in the GC-reactivating, reductase direction. *In vitro*, the functional consequence of this is that inert 11-dehydrocorticosterone (A) was converted to active corticosterone (B), which augmented phagocytic capacity for apoptotic neutrophils (aPMNs). Upon induction of sterile peritonitis, there was a dramatic up-regulation of 11β-HSD1 activity within the cells resident to, or recruited to, the peritoneal cavity. For peritonitis to resolve, aPMNs within the peritoneal cavity must be cleared by Mϕs. In 11β-HSD1-deficient Mϕs, A failed to promote phagocytosis, and phagocytic competency was attained 24h later than WT Mϕs. *In vivo*, 11β-HSD1-deficient mice showed a clearance deficiency of aPMNs, suggesting a physiological role of 11β-HSD1 in promoting clearance of apoptotic cells.

This is the first report in which local modulation of an endogenous factor has been shown to influence apoptotic cell fate.

DECLARATION

This thesis and the data presented in it are entirely the result of my own efforts. This work contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution and, to the best of my knowledge contains no material previously published or written by another person, except where stated in the text.

on ..30/8/..2002.

James Stewart Gilmour

Table of contents

Index of Figures

CHAPTER 1: INTRODUCTION

CHAPTER 2: MATERIALS AND METHODS

CHAPTER 3: 11β-HSD1 IS EXPRESSED IN MACROPHAGES AND MESANGIAL CELLS, AND IS ACTIVE AS A REDUCTASE

CHAPTER 4: CONSEQUENCES OF 11β-HSD1 ACTIVITY *IN VITRO*

CHAPTER 5: MODULATION OF 11β-HSD1 ACTIVITY

CHAPTER 6: CONSEQUENCES OF 11β-HSD1 DEFICIENCY *IN VIVO*

APPENDIX 1: TIME-COURSE OF STERILE PERITONITIS

Index of Tables

CHAPTER 1

CHAPTER 2:

CHAPTER 3:

CHAPTER 6:

Abbreviations

11β-HSD1	11β-hydroxysteroid dehydrogenase 1
11β-HSD2	11β-hydroxysteroid dehydrogenase 2
^3H	tritium
A	11-dehydrocorticosterone
ACTH	adrenocorticotrophic hormone
AP-1	activating protein 1
aPMN	apoptotic PMN
AVP	arginine vasopressin
B	corticosterone
BMD	bone marrow-derived
BSA	bovine serum albumin
CBG	corticosteroid binding globulin
cDNA	complementary deoxyribo-nucleic acid
CM	chloromethyl fluorodiamine (CMFDA)
CNS	central nervous system
CR	complement receptor
Cx	carbenoxolone
DEPC	diethylpyrocarbonate
Dex	dexamethasone
DMEM	Dulbecco's modification of Eagle's Medium
DMSO	dimethylsulfoxide
DNA	deoxyribo-nucleic acid
E	cortisone
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme linked immuno-sorbent assay
EtOH	ethanol
F	cortisol
F12	Ham's F12 medium
FACS	Fluorescence activated cell sorter
Fc	the constant region of immunoglobulin
FCS	foetal calf serum
FSC	forward scatter
GC	glucocorticoid
GR	glucocorticoid receptor
GRE	glucocorticoid-responsive element
H_2O_2	hydrogen peroxide
HBSS	Hank's balanced salt solution (without Ca^{2+} or Mg^{2+})
HI	heat inactivated
HPA	hypothalamic-pituitary-adrenal
HSD	hydroxysteroid dehydrogenase

HPLC	high pressure liquid chromatography
IFN-γ	interferon-gamma
IgG	immunoglobulin G
IL-	interleukin-
iNOS	inducible nitric oxide synthase
i.p	intra-peritoneal
Kb	kilobases
KO	knockout
LPS	lipopolysaccharide
Mϕ	macrophage
MC	mesangial cells
M-CSF	macrophage colony stimulating factor
MD	monocyte-derived
MPO	myeloperoxidase
MR	mineralocorticoid receptor
mRNA	messenger ribo-nucleic acid
NAD	nicotinamide adenine dinucleotide
NADP(H)	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor -κB
nGRE	negative glucocorticoid-responsive element
NO	nitric oxide
PBS	phosphate buffered saline (without Ca^{2+} or Mg^{2+})
PCR	ploymerase chain reaction
PE	phycoerythrin conjugate
PEPCK	phosphoenolpyruvate carboxykinase
PMN	neutrophil
POMC	pro-opiomelanocortin
PRPDS	platelet-rich plasma-derived serum
PS	phosphatidylserine
RNA	ribo-nucleic acid
RNase	ribo-nuclease
RP	resident peritoneal
RT-PCR	reverse transcriptase PCR
SD	standard deviation
SEM	standard error of the mean
SDS	sodium dodecyl (lauryl) sulphate
SSC	side scatter
TE	thioglycollate-elicited
TEP	thioglycollate-elicited peritoneal
TGF-β	transforming growth factor-beta
TNF-α	tumour necrosis factor-alpha

UV	ultra violet
WBC	white blood cells

Awards, Presentations and Publications

SCHOLARSHIPS AND AWARDS

Wellcome Trust Prize Studentship- Four Year PhD studentship programme in the Cellular and Molecular Basis of Disease.

MMC Annual Symposium poster presentation award, 2001.

PRESENTATIONS OF WORK FROM THIS THESIS

Keystone Symposia: Macrophage activation and deactivation. 'Glucocorticoids, 11β–HSDs and Macrophage Function', 2001.

MMC Annual Symposium: '11β–HSDs and Phagocytosis', 2001/2002.

Association of Physicians: 'Glucocorticoid Potentiation of Macrophage Clearance of Cells Dying by Apoptosis', 2001.

The Wellcome Trust Annual Symposium: 'Glucocorticoids, 11β–HSDs and Macrophage Function', 2001/2002.

5th World Congress in Inflammation: '11β–HSD1 and Macrophage Function', 2001.

PUBLICATIONS OF WORK FROM THIS THESIS OR FROM COLLABORATORY WORK

Gilmour, J. S, K. E. Chapman, et al. Local amplification of glucocorticoids is important in determining apoptotic cell fate. Due to be submitted for publication to J. Exp. Med in Sept 2002.

Acknowledgements

COLLEAGUES AT THE MMC. IN PARTICULAR:

Dr. Karen Chapman for expert supervision. The credit for many of the concepts explored throughout this thesis must be given to Karen, whose experience and willingness to supervise me is ultimately responsible for the quality of the work and the early submission of this thesis.

Professor Jonathan Seckl for his ideas, helpful discussions and enthusiasm, which have kept me focused and motivated throughout my studies.

Dr. Moffat Nyirenda for introducing me to endocrinology and Dr. Hayley Harris for the initial study that led to the conception of my project.

Dr. Maggie Lai for assistance with molecular techniques.

Helen Munn for taking on my RT-PCR duties.

David O'Regan, Carina Hibberd and Clare Christy for shadowing me through various protocols with which I was unfamiliar.

Val Lyons for the generation and provision of the 11β-HSD1 probes and primers that made life so easy.

COLLEAGUES AT THE CIR. IN PARTICULAR:

Dr. Simon Brown for early morning supervision, helpful discussion, expert advice and friendship, which have greatly enhanced my enjoyment of my studies.

Professor John Savill for his interest and close involvement in directing my studies. John has generously included me within groups and discussions during which I have had the privilege of meeting and working with many interesting people.

Dr. Adam Lacy-Hulbert, Dr. Jeremy Duffield and Dr. Jeremy Hughes for informative discussions, practical assistance and provision of materials.

Dr. Yuri Kotelevtsev, Dr. Janice Paterson and Professor John Mullins for the generation, backcrossing and up-keep of the 11β-HSD1-deficient mice.

Spike Clay, Dr. Mark Lucas, and Dr. Graham Thomas for technical assistance during my studies. It must not be over-looked that Spike has been crucially involved in every animal experiment (some of which have been out of hours) and has provided highly skilled technical assistance as well as compassionate animal care. His contribution to the completion of this work has been immense.

Dr. Adriano Rossi and Dr. Jean-Michel Sallenave for introducing me to immunobiology.

I am grateful to Clive McKimmie who, under my supervision, assisted in completing and scoring the MC phagocytosis assays over the course of a 10 wk project.

I have enjoyed collaborating with others on work out-with the scope of this thesis. In particular:

Meera Nair and Dr. Judy Allen in exploration of the alternatively activated Mϕ.

Dr. Maria Febbraio and Professor Roy Silverstein for providing me with the opportunity of travelling to NY and studying the CD36-deficient mouse.

Dr. Nik Morton for a short collaboration studying Mϕ uptake of lipoproteins.

Finally, I would like to acknowledge that whilst in John and Jonathan's care I have had the support and encouragement to develop my research ideas and my career plans, for which I am indebted. I sincerely hope that my career allows me the opportunity of future collaboration so that I can repay this kindness.

Chapter 1:

Introduction

1.1 A GLOBAL PERSPECTIVE

Glucocorticoid (GC) therapies are widely used to treat inflammatory diseases, but can be limited by adverse side effects. They act through multiple mechanisms affecting the tissue distributions, effector functions, proliferation and survival of inflammatory cells. GCs are now believed to play a physiological role as well as a pharmacological role in the regulation of inflammation, and such study is opening up new therapeutic approaches to the treatment of inflammatory diseases.

Target cell responses to GCs are controlled by the concentration of circulating adrenal GC and the cellular density of intracellular receptors, of which the glucocorticoid receptor (GR) is the predominant form in most immune tissues. Recent evidence suggests that key modulation of GC action can occur by pre-receptor metabolism of GCs by 11β-hydroxysteroid dehydrogenases (11β-HSDs) (reviewed in (Seckl and Walker 2001)). Two 11βHSD iso-enzymes have been characterised; 11β-HSD2 is an exclusive dehydrogenase that rapidly inactivates GCs preventing binding of GCs to corticosteroid receptors. In contrast, 11β-HSD1 is a reductase in many intact cells *in vitro*, regenerating GCs from inactive 11keto-forms, thus amplifying GC action in specific tissues (Low, Chapman et al. 1994; Jamieson, Chapman et al. 1995; Kotelevtsev, Holmes et al. 1997). Although the role of the 11β-HSDs in metabolic tissues and the CNS are being elucidated, their role in GC modulation in immune tissues, especially in macrophages (Mϕs), has been poorly addressed.

The inflammatory response is a host defence mechanism that evolved in order to kill invading pathogens and repair damaged tissues. It can resolve and restore tissue function, but can become undesirably persistent in a number of disease states. The selective recognition and subsequent phagocytosis of apoptotic neutrophils (aPMNs) by Mϕs is crucial for resolution of inflammation (Savill, Wyllie et al. 1989). Failure to adequately clear aPMNs results in the release of pro-inflammatory mediators and is associated with persistent inflammation, such as glomerulonephritis (Botto, Dell'Agnola et al. 1998; Taylor, Carugati et al. 2000). Recent studies demonstrate a role for GCs,

acting via GR, in promoting the safe clearance of aPMNs by Mϕs (Liu, Cousin et al. 1999).

1.2 GLUCOCORTICOIDS

GCs have a diverse range of physiological functions. Their action is characterised by the stress response – a culmination of both an anticipatory and a reactive adaptation to the environment. Metabolism, immune function, homeostasis and behaviour are all responsive to GC action however, it is essential that GC secretion is regulated to minimise the detrimental effect of inappropriate exposure (reviewed in (De Kloet, Rosenfeld et al. 1988)).

1.2-1 Synthesis:

GCs are steroid hormones predominantly synthesised in the zona fasiculata/ reticularis of the adrenal cortex. There is however, evidence that *in vitro,* they may also be synthesised by cells within the thymus, although this is still controversial (Vacchio, Papadopoulos et al. 1994). All steroids are derived from cholesterol; containing a cyclo-pentane ring and 3 cyclohexane rings (Fig.1-1). Each steroid is synthesised by sequential enzymatic action - predominantly by hydroxylases located in either mitochondria or smooth endoplasmic reticulum. Since rodents lack 17α-hydroxylase in the adrenals, the major GC is corticosterone (B), whilst in humans, cortisol (F) predominates. The adrenals do not store GC, thus it is synthesised *de novo* and released when required. The mineralocorticoid, aldosterone is also synthesised in the adrenal gland and acts primarily in the kidneys to control solute homeostasis (De Kloet, Rosenfeld et al. 1988).

1.2-2 Regulation:

Neuro-endocrine signals, acting via the hypothalamic-pituitary-adrenal (HPA) axis stimulate the production of GCs from the adrenal cortex. A variety of inputs can stimulate the hypothalamus to produce corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP), which are then transported to the pituitary. In response,

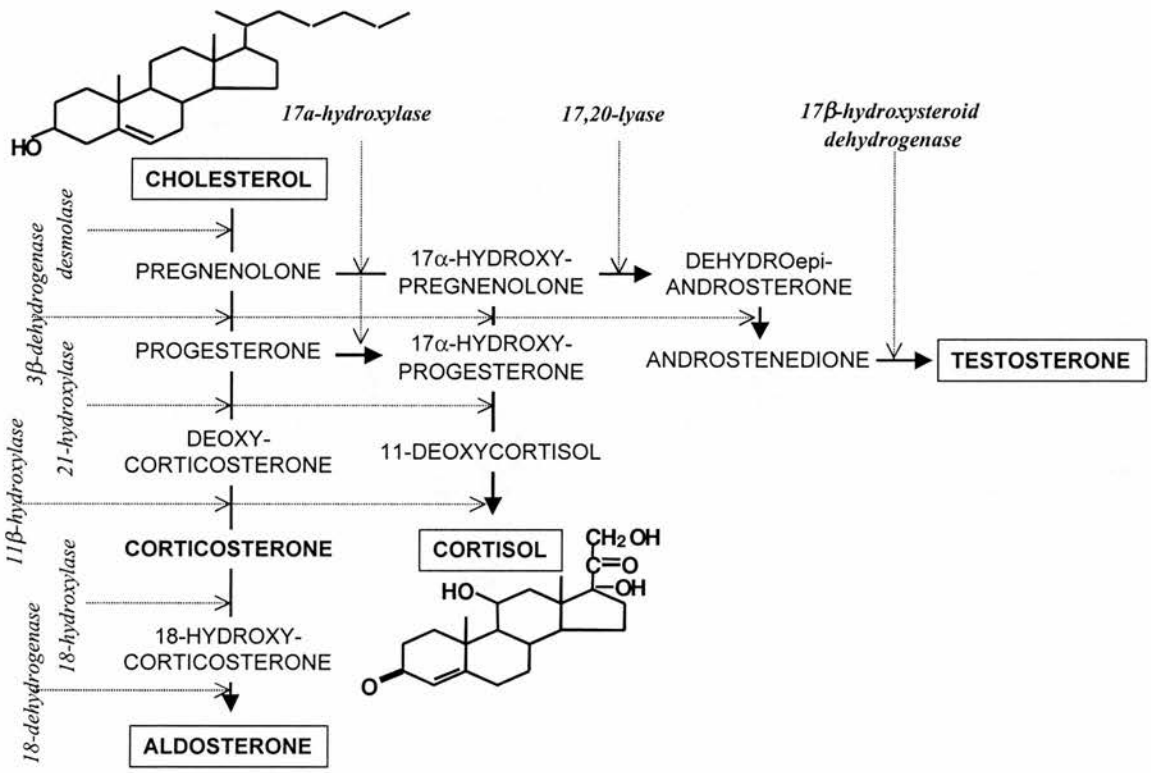


Figure 1-1: The steroid hormones. GCs (cortisol and aldosterone) are steroid hormones for which cholesterol is the common precursor. Diagram adapted from (O'Riordan JLH)

adrenocorticotrophic hormone (ACTH) is rapidly released into the systemic circulation and stimulates the synthesis of GCs and other adrenocorticosteroids from within the adrenal cortex (Axelrod and Reisine 1984). GCs regulate their own suppression via a negative feedback loop in which the circulating concentrations of GCs inhibit the production of CRH and ACTH (Fig.1-2).

GC biosynthesis is also subject to a diurnal rhythm, which peaks at or just prior to the start of the active period (morning for humans, evening for rodents) and is under control of the suprachiasmatic nucleus of the hypothalamus (reviewed in (Dallman, Strack et al. 1993)).

To exert an action, lipophilic GCs cross the membrane of target cells and bind to intracellular cytosolic receptors – GR or mineralocorticoid receptors (MR). Whether GCs freely diffuse across cell membranes or are actively transported is still open to question (Kralli, Bohen et al. 1995). Most mammalian cells express GR, although heterogeneity in GR expression between cell types is observed (Miller, Spencer et al. 1998). MR expression largely is restricted to mineralocorticoid target tissues (including kidney, colon and salivary gland). In the absence of 11β-HSD2 (refer to section 1.3-2), GCs can bind to both GR and MR, with the latter being the higher affinity interaction.

Ligand-free GR is normally bound by cytosolic heat shock proteins (HSP), which dissociate upon ligand binding (Pratt 1993), then the ligand-receptor complex translocates to the nucleus, dimerises, and binds specific regions of exposed DNA where it acts as a transcription regulator (reviewed in (Beato 1989)). Induction of GC-responsive genes normally occurs by binding of GR dimers to palindromic glucocorticoid response elements (GRE) in promoter regions of target genes (Fig.1-2). Alternatively, GR can bind to negative-GREs (nGRE) leading to repression of the transcription of specific genes. However, gene repression may also occur indirectly through the *trans*-repression of transcription factors (reviewed in (Beato 1989; Beato, Chavez et al. 1996)). In addition to direct effects on transcription initiation, the GC-GR complex has also been shown to recruit chromatin-remodelling complexes in order to achieve transcriptional activation (Truss, Bartsch et al. 1995; Fryer and Archer 1998).

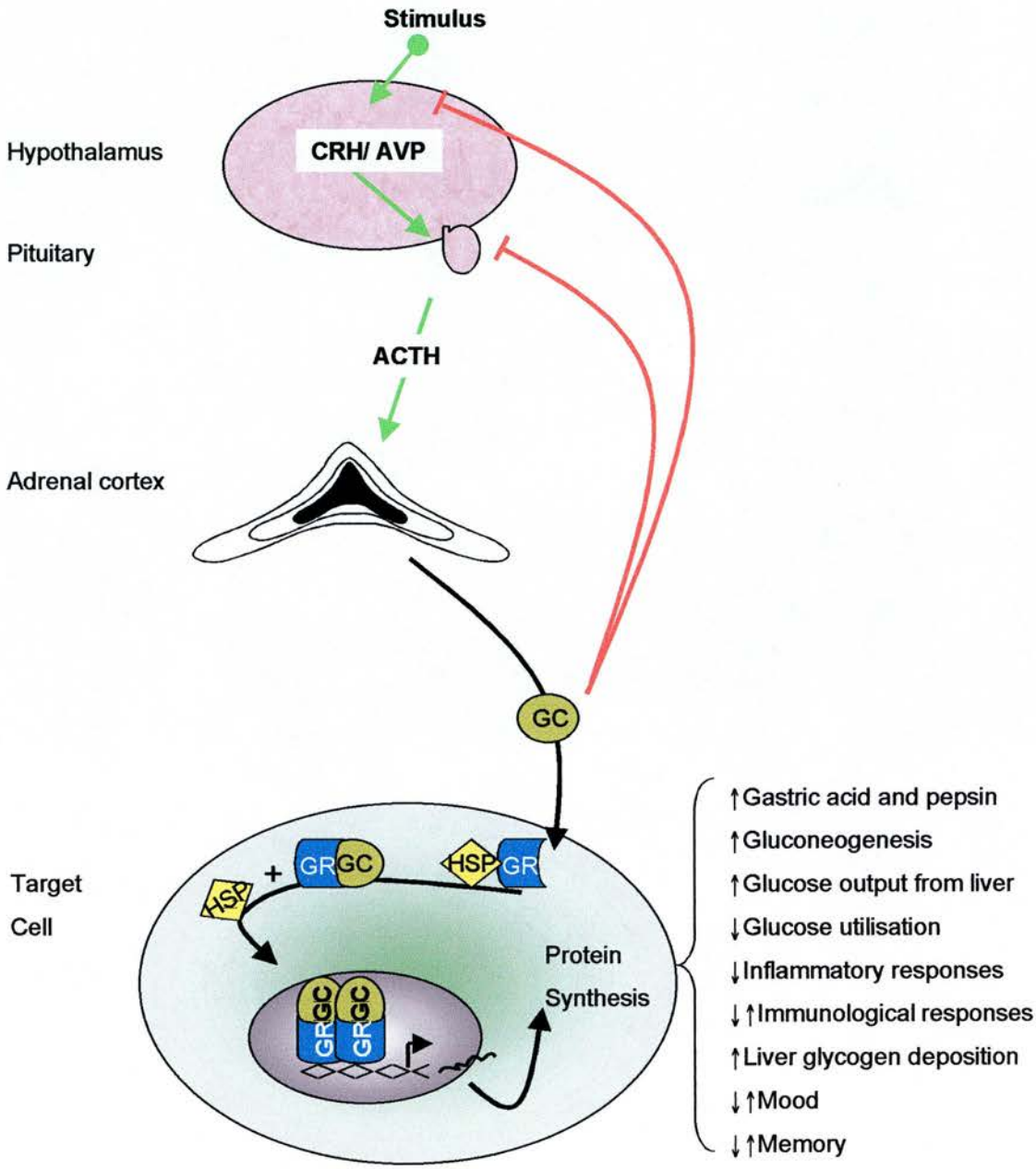


Figure 1-2: A classical view of GC action and regulation by the HPA axis.

Many of the metabolic effects of GCs are due to direct GRE-mediated gene induction, whereas repression of inflammatory genes is often a result of GR/ transcription factor associations. Most commonly GR interfere with activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) function thus preventing their activation of inflammatory response genes (reviewed in (Barnes 1998)). The mechanism of repression is still open to debate. Reichardt et al generated mice with GRs that could not bind DNA due to a dimerisation mutation yet GCs still retained the capacity to repress both AP-1 and NF-κB function in these mice (Reichardt, Kaestner et al. 1998). However, whether the dimerisation mutation completely abrogated DNA binding is questionable, and residual levels of DNA binding may be sufficient to mediate repression.

1.2-3 GC actions:

Much of our knowledge about GC action comes from clinical observation of adrenal malfunction, pharmacological GC treatment and use of GR antagonists. GC excess and deficiency manifest themselves in the conditions Cushing's syndrome and Addison's disease, respectively. The first is characterised by insulin insensitivity, truncal obesity, glucose intolerance, hypertension, change in mood, myopathy and increased susceptibility to infection. Conversely, Addison's disease leads to hypoglycaemia, hyperpigmentation, anorexia, and impaired kidney function (hypotension and hyponatraemia). Such syndromes are rare, however it is believed that subtle yet chronic alterations in GC metabolism can play a causative role in common disorders such as obesity, hypertension and diabetes (Seckl and Walker 2001).

One of the best established effects of GC is that of inducing apoptosis of thymocytes, *in vitro* - an effect that requires high levels (10^{-7}M-10^{-6}M). Stress levels of GC are enough to cause thymus involution, *in vivo*, suggesting that this effect is physiologically relevant. Immature thymic T-cells have been found to express high levels of GR, consistent with GC-sensitivity, however mature splenic T-cells are less sensitive to GC and accordingly have been shown to express lower levels of GR (Miller, Spencer et al. 1998). GC-mediated selection of immature thymic T cells has been suggested to be accomplished by synergistic action between GC and T-cell receptor (TCR) ligation

(Jamieson and Yamamoto 2000). T cell survival is determined by the avidity of the TCR for self-antigen (and possible auto-immunity) or major histocompatibility complex (and adaptive immunity) (reviewed in (Janeway CA 1999)). Only simultaneous signalling through GR and TCR-mediated pathways ensures survival through a, as yet unclear, mechanism dependent on GR density and TCR avidity for auto-antigens (reviewed by (Ashwell, King et al. 1996)). Interestingly, it has been proposed that the GC synthesised in the thymus itself (thymic epithelium) facilitates this selection (Vacchio, Papadopoulos et al. 1994). Furthermore, studies by Vacchio et al, which utilised transgenic mice carrying a transgene for TCR, showed that T cells that would normally have survived were deleted by apoptosis after treatment with a GC-biosynthesis inhibitor (Vacchio and Ashwell 1997).

However, this subject is still controversial, since mice which lack GR have an apparently normal thymus at birth (Purton, Boyd et al. 2000). The lack of GR causes neo-natal lethality, precluding analysis of the adult phenotype (Purton, Boyd et al. 2000), and thus the role of GR in T-cell selection remains to be resolved.

1.2-4 Anti-inflammatory effects:

GCs are rapidly induced during the stress response, in which stimuli to the hypothalamus increase CRH release (Munck, Guyre et al. 1984). The source of stress varies, but can include the direct action of pro-inflammatory cytokines upon cells of the hypothalamus or pituitary, or the adrenal cortex itself (Mandrup-Poulsen, Nerup et al. 1995). The absolute importance of adrenal GC action during inflammation can be crudely demonstrated by adrenalectomy and subsequent challenge with LPS or TNFα (Bertini, Bianchi et al. 1988). Animals that were previously tolerant to moderate doses of LPS or TNFα die if adrenalectomised; however they survive if administered GCs.

Despite the side effects of GC excess, GC treatments are established therapies for a number of acute and chronic inflammatory conditions including asthma and arthritis. Although their anti-inflammatory actions at the cellular and molecular levels are yet to be completely elucidated, it is clear that they exert a multifaceted action on many immune cell types, in particular the GC-sensitive leukocytes - monocytes, Mϕs and

lymphocytes (Miller, Spencer et al. 1998). However, extensive study of GC effects has produced a somewhat confused literature; subject to dosage, species, *in vitro* and *in vivo* anomalies. With increasing understanding of cellular processes these issues are gradually being resolved and it is clear that the pleiotropic effects of GCs upon leukocyte gene transcription can alter protein synthesis, processing and secretion, as well as cell growth, division and apoptosis (Goulding and Guyre 1993). GCs have been shown to repress the expression of cytokines, adhesion molecules, receptors and enzymes to produce an overall effect of immune suppression. This can include the inhibition of leukocyte circulation, recruitment and infiltration, and inhibition of pro-inflammatory mediator release by monocytes/ Mϕs (reviewed in (Schleimer 1993; Barnes 1998)).

As alluded to in section 1.2-2, many target genes suppressed by GCs have no GRE or nGRE within promoter regions, but instead have binding sites for transcription factors AP-1 and NF-κB (Cato and Wade 1996). AP-1 and NF-κB are potent inducers of pro-inflammatory cytokines, and their actions are antagonised by GCs. The mechanisms by which the GC-GR complex interferes with the binding of AP-1 and NF-κB is unclear, however, it is possibly through direct binding interactions or through the induction of inhibitory intermediary molecules (such as IκB-α) (Ray and Prefontaine 1994; Auphan, DiDonato et al. 1995). Some of the myriad of GC-mediated transcriptional effects on immune cells are summarised in Table 1-1.

Whilst pharmacological doses of GCs are immunosuppressive, there is growing realisation that physiological levels of endogenous GCs are immunomodulatory rather than solely immunosuppressive. Prolonged stress, leading to chronic adrenal activation can increase susceptibility to, or severity of, infection whereas a blunted adrenal response can predispose towards inflammatory, autoimmune and allergic diseases (Sternberg 2001). The innate immune system is largely regulated by Mϕs, whilst the adaptive response is regulated by T-helper lymphocytes (Th1 and Th2 subclasses). Although the distinction between Th1 and Th2 populations is less clear than previously thought, physiological concentrations of GCs are known to shift cytokine production by

Increased Transcription	Decreased Transcription	Cellular Effects
IL-4, -5, and -10, TGF-β, MIF, lipocortin-1, IL-1 receptor agonist, IκB-α.	IL-1, -2, -3, -6, -8, -11, -12, and -13, TNFα, IFN-γ, GM-CSF, RANTES, iNOS, MCP-1, -2, -3, and -4, phospholipase A_2, ICAM-1, elastase, plasminogen activator, cyclooxygenase.	Suppression of leucocyte adhesion, migration and infiltration. Reduction in numbers of circulating monocytes and inhibition of Mϕ activation, mediator release and antigen presentation. Suppression of lymphocyte activation and proliferation, and induction of apoptosis. Inhibition of antibody production.

Table 1-1. Effects of GC on immune cells. Adapted from (Lee, Tsou et al. 1988; Schleimer 1993; Barnes 1998).

lymphocytes from the largely pro-inflammatory Th1 profile to the anti-inflammatory Th2 profile (Elenkov and Chrousos 1999). The Th1 response is co-ordinated by Mϕ activation and is characterised by the production of IL-2 and IFN-γ, whilst a Th2 response is associated with an adaptive response, characterised by IL-10 and IL-4 production (reviewed in (Elenkov and Chrousos 1999)). Therefore GC function may not be to necessarily defend against the pathogen, but rather to act as a physiological brake by limiting the tissue damaging potential of Mϕs and other Th1 cells.

1.2-5 Access of glucocorticoids to the glucocorticoid receptor:

Access of GCs to the GR can be controlled at various levels. Firstly, as detailed above, release of adrenal-GC into the systemic circulation is regulated by HPA activity, subject to control by negative feedback. However it is believed that 95% of endogenous circulating GC is bound to corticosteroid binding globulin (CBG) and is therefore unavailable as a ligand for GR. Apart from drastically reducing the concentration of GC made available to cells, CBG may actually be a GC delivery mechanism to cells in a manner which ensures equal distribution of ligand to peripheral GC-target cells (Pardridge 1987). Release of GC from CBG can be influenced by environmental factors rather than the HPA – for example dissociation has been shown to occur following proteolytic cleavage by elastase released by activated PMNs (Hammond, Smith et al. 1990).

Secondly, the density of GR in target tissues is a major determinant of cellular sensitivity to GC. Lack of GR causes neo-natal lethality in transgenic mice (Finotto, Krieglstein et al. 1999). *In vitro*, GR expression is proportional to GC sensitivity (Vanderbilt, Miesfeld et al. 1987), and the expression of GR anti-sense RNA *in vivo*, which causes a 30-50% reduction in GR levels, leads to GC resistance in mice (Pepin, Pothier et al. 1992). Syndromes of GC resistance have been identified in patients with rheumatoid arthritis (Poznansky, Gordon et al. 1984) and asthma (Alvarez, Surs et al. 1992; Adcock, Lane et al. 1995) and there is evidence that limiting concentrations of GR determine the GC-sensitivity of target cells. For instance, in GC-resistant rheumatoid arthritis, GR levels in lymphocytes can be reduced by up to 50% (Poznansky, Gordon et

al. 1984; Chikanza, Petrou et al. 1992), and in cases of GC-resistant asthma, decreased GR expression in leukocytes is associated with reduced GC-suppression of cytokine release from monocytes (Alvarez, Surs et al. 1992; Chikanza, Petrou et al. 1992; Lane, Wilkinson et al. 1993; Adcock, Lane et al. 1995). GR levels appear to be negatively regulated in an autocrine fashion by the levels of circulating GC, which increase with chronic stress and GC therapy (Makino, Smith et al. 1995; Kitraki, Karandrea et al. 1999), and in some cases with age (Sapolsky, Krey et al. 1984).

An additional possible modulator of GC function is an alternatively spliced isoform of GR, GRβ, which cannot bind ligand due to a truncated C-terminus. It has been suggested that GRβ is constitutively expressed and acts as a dominant negative inhibitor of the full length GR (GRα), although its action has only been demonstrated, *in vitro* to date (Bamberger, Bamberger et al. 1995). However, since GRβ is not expressed in rodents (the splice site is not conserved) its physiological significance remains questionable (Hecht, CarlstedtDuke et al. 1997; Otto, Reichardt et al. 1997).

Thirdly, non-genomic effects of steroids have been described (reviewed in (Losel and Wehling 2003)) and whilst there is evidence for a rapid, non-genomic cellular response to progesterone through a membrane-associated receptor (reviewed in (Bramley 2003)) there is, as yet, little convincing evidence that similar mechanisms exist for GCs. In fact, one might expect to find a percentage of the cell's GR associated with the membrane as it sequesters its lipophilic ligand.

Lastly, recent evidence suggests that key modulation of GC action can occur by pre-receptor metabolism of GCs by 11β-hydroxysteroid dehydrogenases (11β-HSDs) (Seckl and Chapman 1997). Two 11β-HSD iso-enzymes have been characterised; 11β-HSD1 and 11β-HSD2.

1.3 11β-HYDROXYSTEROID DEHYDROGENASES

1.3-1 History:

Amelung described the inter-conversion of GCs (B and F) and their inert 11-keto forms (A and E) in the liver in 1953 (Amelung D 1953). This activity was later characterised and purified by Monder et al in 1985, and was believed to represent a route of GC clearance from the circulation. (Lakshmi and Monder 1985; Lakshmi and Monder 1985; Lakshmi and Monder 1988). The importance of 11β-HSD was subsequently realised when its action was found to provide an explanation to the 'mineralocorticoid receptor paradox' of the kidney; namely, that MR sites within the distal nephron bind both GCs and mineralocorticoids *in vitro*, but are mineralocorticoid-specific *in vivo*. Therefore the inactivation of GCs by 11β-HSD activity was credited with this *in vivo* selectivity, particularly once it had been shown that 11β-HSD inhibition with liquorice-derivatives allowed the non-selective and inappropriate binding of GCs to MRs, leading to hypertension (Stewart, Valentino et al. 1987; Funder, Pearce et al. 1988). Therefore the specificity of mineralocorticoid target tissues is enzyme mediated, not receptor mediated.

However it was found that polyclonal Abs raised against 11β-HSD purified from liver unexpectedly stained the kidney proximal tubules, not the MR-rich distal nephron area (Agarwal, Monder et al. 1989). Subsequently a second iso-enzyme with different kinetics and tissue distribution, 11β-HSD2, was purified and cloned (Agarwal, Mune et al. 1994; Albiston, Obeyesekere et al. 1994; Brown, Chapman et al. 1996; Brown, Chapman et al. 1996). 11β-HSD1 and 11β-HSD2 are distantly related members of the short chain alcohol dehydrogenase family, sharing only approximately 20% identity, but which have conserved regions of co-factor binding and active site (reviewed in (Stewart and Krozowski 1999)). Figure 1-3 contrasts the biological roles of the 11β-HSDs.

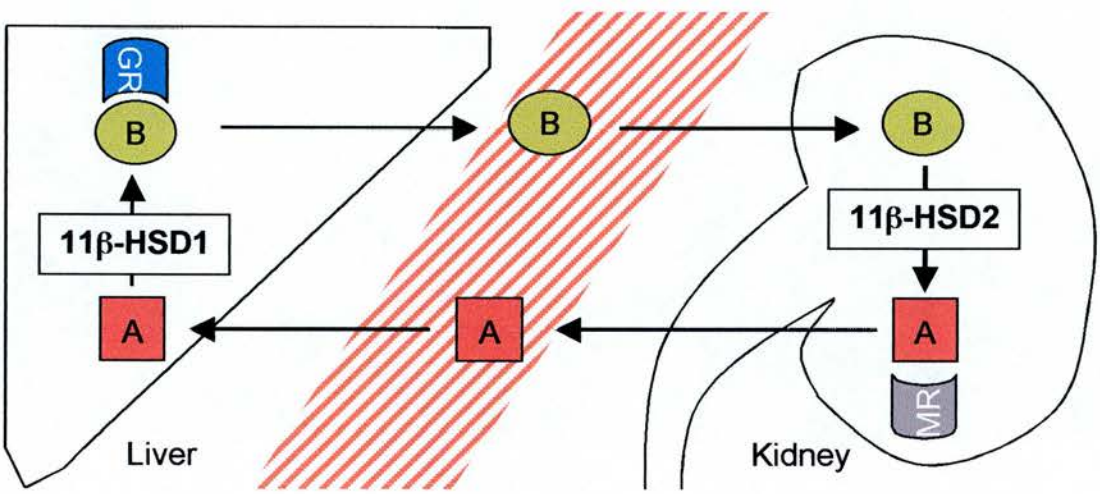


Figure 1-3: Contrasting roles of the 11β-HSDs. 11β-HSD2 is an exclusive dehydrogenase that acts in aldosterone target tissues (eg. kidney) to exclude B from otherwise non-selective MR. 11β-HSD1 is a reductase that acts in predominantly GC-target tissues (eg. liver) to amplify intracellular B concentrations. Figure adapted from (Seckl and Walker 2001).

1.3-2 11β-HSD2:

11β-HSD2 is a high affinity, NAD-dependent enzyme that, through 11β-dehydrogenase action, inactivates GCs thus preventing access to otherwise non-selective MR in aldosterone target tissues such as the distal nephron, and in placenta where it protects against detrimental over-exposure to maternal GCs (Table.1-2) (Edwards, Stewart et al. 1988; Funder, Pearce et al. 1988; Krozowski, MaGuire et al. 1995; Seckl, Benediktsson et al. 1995). This can be demonstrated *in utero* where 11β-HSD2 inhibition or chronicadministration of GCs to pregnant rats programs the pups to have lowered birth weights and increased risk of hypertension (Benediktsson, Lindsay et al. 1993; Lindsay, Lindsay et al. 1996).

1.3-3 11β-HSD1:

11β-HSD1 has a lower affinity for GC than 11β-HSD2, is NADP(H)- dependent and is widely expressed, with highest levels in classical GC-target tissues where it probably functions as an 11β-reductase, reactivating B from inert A (F from E in humans) (Table.1-2) (Jamieson, Chapman et al. 1995; Kotelevtsev, Holmes et al. 1997). Purified 11β-HSD1 acts as a dehydrogenase, but in intact cells 11β-HSD1 cDNA encodes 11β-reductase activity (Agarwal, Monder et al. 1989; Low, Chapman et al. 1994). Furthermore, depending on the steroid substrate and co-factor present, 11β-HSD1 activity has been shown to be bi-directional in tissue homogenates - thus it is clear that the direction of 11β-HSD1 activity is cell context dependent (Lakshmi and Monder 1988). Most evidence suggests that the *in vivo* activity is that of a reductase (Low, Chapman et al. 1994; Kotelevtsev, Holmes et al. 1997; Jamieson, Walker et al. 2000), and that the enzyme is located within the lumen of the endoplasmic reticulum (ER) (Ozols 1995). Figure 1.4 shows the inter-conversion of A and B by 11β-HSD1.

Although in normal physiology plasma A levels are substantially lower than B levels, (because 95% of B is bound by CBG) the level of A is similar to, if not greater than, that of free B (Harris, Kotelevtsev et al. 2001). A is generated by 11β-HSD2 action (mainly

	11β-HSD1	11β-HSD2
Reaction direction in intact cells	Reductase	Dehydrogenase
Co-factor	NADP(H)	NAD(H)
Size	34 kDa	40 kDa
K_m for steroid substrate	120nM for A	14nM for B
Sites of expression	Widespread, including liver, kidney (proximal tubules), adipose tissue, brain, lung, pituitary, gonads, skeletal muscle and vascular smooth muscle. Most recently, mesangial cells and monocytes.	Kidney (distal tubules), colon, salivary glands, sweat glands, vascular endothelium and placenta.
Phenotype of 11β-HSD-deficient mice	Altered blood glucose after over feeding or stress, impaired activation of gluconeogenesis on fasting, hypercorticosteronaemia, protection from age-associated cognitive decline, raised levels of high-density lipoprotein cholesterol, and lowered levels of low-density lipoprotein cholesterol and triglycerides.	Severe hypertension, hypokalaemia, renal structural abnormalities, increased risk of post-natal death.

Table 1-2. Characteristics of 11β-HSD1 and 11β-HSD2. 11β-HSD1 data from (Kotelevtsev, Holmes et al. 1997; Harris, Kotelevtsev et al. 2001; Morton, Holmes et al. 2001; Yau, Noble et al. 2001). 11β-HSD2 data from (Brown, Chapman et al. 1993; Kotelevtsev, Brown et al. 1999). Table adapted from (Sandeep and Walker 2001).

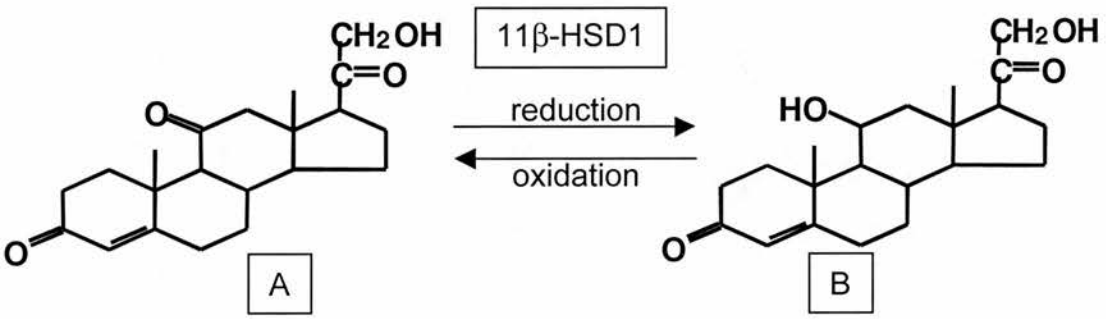


Figure 1-4: The inter-conversion of A and B by 11β-HSD1. 11β-HSD1 can catalyse the inter-conversion of inert 11-dehydrocorticosterone (A) and active corticosterone (B). Cortisone and cortisol differ from A and B due to the presence of a hydroxyl group at position C-17.

in the kidney) and in mice it circulates at concentrations of approximately 5nM without being subject to a diurnal rhythm (Whitworth, Stewart et al. 1989; Harris, Kotelevtsev et al. 2001). Therefore a sufficient pool of A circulates to provide a means to amplify intracellular B concentrations in specific cells in order, for example, to maintain glucose homeostasis during circadian rhythm or enhance the stress response (Seckl and Chapman 1997).

1.3-4 11β-HSD1 deficiency:

Recent clinical observations have attributed the failure of a Cushing's patient to present with a 'Cushingoid' phenotype to a partial defect in 11β-HSD1 activity (Tomlinson, Draper et al. 2002). Failure to regenerate F from E, led to a relative increase in the metabolic clearance of F, and thus protected the patient from the effects of excess GC (Tomlinson, Draper et al. 2002). There is also evidence of 11β-HSD1 dysregulation in a small number of patients with syndromes linked to androgen excess and in primary obesity, although no gene mutations have yet been described (reviewed in (Seckl and Walker 2001)). However, in mice the evidence suggests that 11β-HSD1 deficiency attenuates the normal increases in expression of gluconeogenic enzymes and hyperglycaemia seen upon stress or obesity (Table.1-2) (Kotelevtsev, Holmes et al. 1997). Following stress, levels of A in plasma 11β-HSD1-deficient mice rise to levels comparable to B, suggesting that under conditions where the HPA axis is activated conversion of A to B by 11β-HSD1 may contribute substantially to the activation of GRs (Harris, Kotelevtsev et al. 2001).

Additionally, recent work shows that inability to regenerate B confers protection from age-associated cognitive decline (Yau, Noble et al. 2001). 11β-HSD1-deficient mice hyper-secrete B, presumably to compensate for the inability to regenerate B from A (Kotelevtsev, Holmes et al. 1997). Intriguingly the high plasma B levels in 11β-HSD1-deficient mice are not reflected in brain tissue, which, in fact, has lower B levels than wild type (WT) mice (Yau, Noble et al. 2001), suggesting that some tissues are largely dependent on 11β-HSD1 action for their source of B.

1.4 INFLAMMATION

Inflammation was first described centuries ago as redness and swelling, with heat and pain. Typically, symptoms are effected by a plethora of chemical mediators, which control haemodynamic changes, vascular permeability and white cell influx in a regulated fashion in the affected tissues. The entry of pathogens into the body induces the release of chemotactic factors, such as prostaglandins, histamine, complement-derived peptides and cytokines, which recruit leukocytes from the bloodstream towards the site of inflammation. To accomplish this, cells have to undergo tethering and adhesion to, and translocation through the endothelial layer of the vessel wall at the appropriate site, and then migrate through the interstitium towards the inflammatory foci. In the peritoneum for example, the resident cell population is almost exclusively resident peritoneal (RP) Mϕs, and during peritonitis, the recruited cell population are predominantly activated PMNs and monocytes (Andrews 1998)- the primary effectors of micro-organism ingestion and killing.

When activated by the inflammatory environment, PMNs release oxygen radicals and proteolytic enzymes such as elastase, which aid the killing of micro-organisms, but also have the capacity to damage host tissues when inappropriately released (Henson and Johnston 1987). Whilst it has been shown that a degree of PMN-mediated tissue damage is necessary for the proper induction of an acute inflammatory response, when this level of control is lost the response is exacerbated by a chronic accumulation of potentially damaging activated PMNs and inflammatory diseases may develop (Henson and Johnston 1987; Malech and Gallin 1987)

Mϕs differentiate and mature from recruited monocytes. Maturation is accompanied by increased secretion of, and sensitivity to inflammatory cytokines, and numerous changes in surface antigens (refer to section 1.4-1) (reviewed in (Janeway CA 1999)). This leads to an enhancement of recognition and phagocytosis of micro-organisms (reviewed in (Greenberg and Grinstein 2002)). However, such changes also equip a dysfunctional Mϕ to perpetuate the inflammatory response through tissue damage.

In the event that regulation of the inflammatory process is lost, resolution cannot occur and inflammation often persists. In order to limit the chance of tissue damage, pathogen removal must be followed by the dissipation of pro-inflammatory mediators, cessation of leukocyte influx and induction of anti-inflammatory mediators. Finally, cell removal or emigration must occur (Haslett 1992). Evidence suggests that extravasated PMNs that cannot be cleared via a return to the bloodstream or incorporation into excreta meet their fate directly at the site of inflammation (Hughes et al. 1997). In contrast, inflammatory Mϕs do not die *in situ*, but emigrate to the draining lymph nodes (Bellingan, Caldwell et al. 1996).

1.4-1 Inflammatory macrophages:

Mϕs influence almost all aspects of the inflammatory response and play a crucial role in linking the innate and acquired arms of immunity. Not only are pathogens phagocytosed and degraded, but also their antigenic components are presented to T cells.

In 1973, van Furth et al showed that inflammatory peritoneal Mϕs were predominantly derived from blood-borne monocytes, and that the increased numbers present after the onset of inflammation were therefore predominantly due to recruitment rather than proliferation *in situ* (Van Furth, Diesselhoff-den Dulk et al. 1973). Inflammatory Mϕs differentiate from blood-borne monocytes, which are themselves derived from precursor monoblasts, resident in the bone marrow. Monoblasts proliferate in the presence of IL-3 and GM-CSF then differentiate into monocytes in the presence of M-CSF. Once a monocyte has left the peripheral blood and entered a tissue, differentiation is triggered (again under the influence of IL-3, GM-CSF and M-CSF which Mϕ themselves can elicit). As Mϕs mature they increase their numbers of mitochondria, induce the production of an array of lysosomal enzymes and reactive oxygen intermediates, up-regulate Fc and complement receptor expression and develop phagocytic capacity (Beelen and Walker 1983; Hamilton 1993; Ogawa 1993). Whilst mature Mϕs retain the ability to proliferate, their function and lifespan are tissue-dependent. For example, splenic Mϕs remove erythrocytes from the blood, in the bone marrow they transfer iron to erythroblasts and in tissues they co-ordinate an inflammatory response in which they

not only induce injury, but also orchestrate resolution and repair (reviewed in (Janeway CA 1999)). The mechanisms by which Mϕ phenotypes are determined are unclear and are likely to be influenced by exposure to growth factors, transcription factors and pro-inflammatory mediators (reviewed in (Valledor, Borras et al. 1998)), including peroxisome proliferator-activated receptorγ (PPARγ) and members of the CCAAT/enhancer binding protein (C/EBP) family (refer to section 1.4-3). For example, Th1 cytokine (IFN-γ) exposure induces differentiating Mϕs towards a pro-inflammatory phenotype, whereas early exposure to Th2 cytokines (IL-4) primes the Mϕ towards a non-inflammatory 'alternative' phenotype (Riches 1995; Erwig, Kluth et al. 1998).

The distinction between resident tissue Mϕs (eg. peritoneal RP Mϕs) and inflammatory Mϕs is less clear. RP Mϕs appear to be quiescent in as much as they have low synthetic activity, low RNA content, low oxygen consumption, little or no cytokine secretion but they do retain phagocytic ability. They are long lived (≥50 days) (Melnicoff, Horan et al. 1988; Melnicoff, Horan et al. 1989), and are thought to be replenished by low level proliferation and immigration from monocytes (Sawyer, Strausbauch et al. 1982).

1.4-2 11β-HSD1 and the immune system:

Previous studies with glycyrrhetinic acid, an 11β-HSD inhibitor have suggested a role for 11β-HSDs in immunity (Finney and Somers 1958). For example glycyrrhetinic acid has been shown to inhibit growth and cytopathology of a number of viruses (Pompei, Flore et al. 1979), an effect that is probably due to the increased GCs associated with the inhibition of 11β-HSD2. Also, vascular permeability to leukocytes is decreased by vasoconstriction after topical administration of GCs, but when combined with glycyrrhetinic acid, this effect is greatly enhanced (Teelucksingh, Mackie et al. 1990). However, despite such circumstantial evidence of 11β-HSD involvement, there are only limited reports of 11β-HSD expression and activity in the immune system.

Hennebold was the first to suggest an immune function for the 11β-HSD isoenzymes. 11β-dehydrogenase activity was detected in homogenates of stromal cells of the lymphoid organs and thus it was postulated to protect immature lymphocytes from the

pro-apoptotic effects of GCs (Hennebold, Ryu et al. 1996). Furthermore, inhibition of this intra-lymphoid 11β-HSD activity was reported to shift cytokine production by activated T lymphocytes from a Th1 to Th2 profile, and increase susceptibility to bacterial disease (Hennebold, Ryu et al. 1996; Hennebold, Mu et al. 1997).

Although not in the immune system, a physiological role for the 11β-HSDs in the regulation of ovulatory cycle has been postulated. Tetsuka et al have shown that 11β-HSD1 and 11β-HSD2 in granulosa cells are reciprocally expressed during the ovulatory cycle, with the expression of 11β-HSD2 prior to ovulation in a "pro-inflammatory phase" (culminating in the release of the oocyte), followed by expression of 11β-HSD1 in an "anti-inflammatory phase" (the tissue repair of the ovary) (Tetsuka, Thomas et al. 1997; Tetsuka, Milne et al. 1999). Additionally, luteinizing hormone and IL-1β have been shown to up-regulate 11β-HSD1 gene expression by granulosa cells *in vitro*, and therefore increased GCs, generated through the amplification of A to B, may have an anti-inflammatory role during ovulation (Tetsuka, Haines et al. 1999).

In 2000, 11β-HSD1 was identified in a subtractive hybridisation screen as induced during the *in vitro* differentiation of a human myelomonocytic cell line (Gingras and Margolin 2000). Later, definitive work by Thieringer described induction of 11β-HSD1 expression in human monocyte-derived (MD) Mϕs (Thieringer, Le Grand et al. 2001).

1.4-3 Regulation of 11β-HSD1:

The transcription of 11β-HSD1 can be modulated by both endogenous and exogenous factors. For example, 11β-HSD1 is up-regulated by GCs (Low, Moisan et al. 1994) and thyroid hormone *in vitro* (Whorwood, Sheppard et al. 1993), and by chronic stress induced by adjuvant arthritis *in vivo* (Low, Moisan et al. 1994). In contrast, there is evidence that down-regulation can be effected by sex steroids (Low, Assaad et al. 1993; Low, Chapman et al. 1994) and insulin (Hammami and Siiteri 1991; Jamieson, Chapman et al. 1995). Hepatic sexual dimorphism of 11β-HSD1 expression is observed in rats but not mice, with females having 2-fold lower 11β-HSD1 expression (Low, Chapman et al. 1994; Rajan, Chapman et al. 1995). This was shown to be due to the continuous (female)

or pulsatile (male) nature of growth hormone secretion, under the control of sex steroids (Low, Chapman et al. 1994).

The idea of 11β-HSD1 as a modulator of GC action within immune cells is an attractive one. Induction of 11β-HSD1 could suffice to amplify GC levels locally within the inflammatory foci. Likely sources of effector, particularly during inflammation, would be the cytokines and other mediators of the inflammatory response. The two phagocyte studies that have been published to date show conflicting results (Escher, Galli et al. 1997; Thieringer, Le Grand et al. 2001). Firstly, studies of mesangial cells (MC), which share some characteristics with Mϕs, demonstrated up-regulation of 11β-HSD1 by the pro-inflammatory cytokines, TNF-α and IL-1β (Escher, Galli et al. 1997). In contrast to this Th1 effect, 11β-HSD1 expression in human MD Mϕs was recently shown to be induced by the Th2 cytokines IL-4 and IL-13; an effect that was abrogated in the presence of pro-inflammatory IFN-γ (Thieringer, Le Grand et al. 2001). In the same study, the potent inflammatory agent LPS was shown to up-regulate 11β-HSD1 expression in the Mϕ-like cell line, THP-1 (Thieringer, Le Grand et al. 2001).

There is an increasing literature on the regulation of adipocyte differentiation, which may show parallels to monocyte differentiation. Increased concentrations of GCs in adipose tissue have been implicated in the pathogenesis of visceral obesity by inducing adipogenesis and increasing adipocyte lipid metabolism (Ringold, Chapman et al. 1986; Hauner, Entenmann et al. 1989; Bujalska, Kumar et al. 1997). Adipose tissue exhibits substantial 11β-HSD1 activity, and 11β-HSD1-deficient mice have been shown to resist insulin resistance and hyperglycaemia (Kotelevtsev, Holmes et al. 1997). In contrast, transgenic mice which over-express 11β-HSD1 in fat, develop visceral obesity and other symptoms of the "metabolic syndrome" (Masuzaki, Paterson et al. 2001). Recently, adipocyte specific induction of 11β-HSD1 by cytokines was shown *in vitro* (Tomlinson, Moore et al. 2001). 11β-HSD1 expression in primary adipocytes was increased by TNFα, IL-1β, leptin and orphan nuclear receptor peroxisome proliferator-γ agonists, but inhibited by IFN-γ.

PPARs are ligand-regulated transcription factors. PPARα and γ have been shown to be induced during monocyte differentiation, and PPARγ during adipocyte differentiation (Brun, Tontonoz et al. 1996; Chinetti, Griglio et al. 1998; Tontonoz, Nagy et al. 1998). In adipocytes, activation of PPARγ induces differentiation and genes involved in lipid metabolism (Rosen, Sarraf et al. 1999), and, in an adipocyte cell line has been shown to negatively regulate 11β-HSD1 expression (Berger, Tanen et al. 2001). Also, PPARα agonists have been shown to reduce 11β-HSD1 expression in liver cells (Hermanowski-Vosatka, Gerhold et al. 2000). Recently associations has been found between PPAR signaling pathways and monocyte differentiation and lipid metabolism, with PPARγ acting as a negative regulator for Mϕ activation (Ricote, Li et al. 1998; Tontonoz, Nagy et al. 1998). It is unknown whether Mϕ 11β-HSD1 expression would be affected by PPAR agonists. However it has been shown that PPAR-γ expression is inducible by IL-4, and negatively regulates some Mϕ pro-inflammatory genes through antagonism of AP-1 and NF-κB mediated gene transcription (Huang, Welch et al. 1999) (Jiang, Ting et al. 1998; Ricote, Li et al. 1998). An exacerbated inflammatory response to LPS seen in PPARα-deficient mice (Delerive, De Bosscher et al. 1999).

Similarly, PPARγ has been shown to co-operate with C/EBPα in the promotion of adipocyte differentiation (Wu, Rosen et al. 1999). Binding sites for C/EBP have been identified within the 11β-HSD1 promoter region, and C/EBPα has been shown to positively regulate 11β-HSD1 expression in hepatoma cells (Williams, Lyons et al. 2000). However, whilst there are no reports of 11β-HSD1 regulation by C/EBP in immune cells, C/EBP binding sites have been located in promoters of monocyte and Mϕ genes such as TNFα and IL-6 (Matsusaka, Fujikawa et al. 1993; Pope, Leutz et al. 1994; Tanaka, Akira et al. 1995), and C/EBPβ has been proposed to be an important regulator of genes involved in inflammation (Poli 1998). Moreover, C/EBP-ε-deficient mice have been shown to have dysfunctional Mϕs (Tavor, Vuong et al. 2002).

1.5 RESOLUTION OF INFLAMMATION

Successful resolution of an inflammatory response is, in part, controlled by the apoptosis (programmed cell death) of granulocytes. Whilst facilitating PMN deletion (and the safe disposal of their histotoxic contents) from the inflamed site, apoptosis is also associated with the functional isolation of the granulocyte from the inflammatory environment by the loss of stimulated chemotaxis, phagocytosis, degranulation and respiratory burst (Haslett, Lee et al. 1991; Whyte, Meagher et al. 1993). The crucial distinction between this form of death and uncontrolled necrosis is that cells dying by apoptosis do not fall apart and liberate their intracellular contents but rather they retain their membrane integrity and disappear without damaging surrounding tissue and inciting further inflammation (Wyllie, Kerr et al. 1980).

1.5.1 Apoptosis:

Wyllie and colleagues first described apoptosis in 1972 (Kerr, Wyllie et al. 1972). Since then it has been found to permeate almost every aspect of cell biology; blastocyst formation, tissue re-modelling during development and after damage, negative selection of lymphocytes, growth and regression of tumours, regulation of haemopoietic cell number and maintenance of all healthy adult tissues that require cell renewal (Vaux and Korsmeyer 1999). Despite its occurrence in many different settings, there is uniformity in the molecular mechanisms and morphology of apoptosis. Typically morphological changes include loss of contact with neighbouring cells, condensation of nuclear chromatin, DNA cleavage by endonucleases, cell shrinkage, Golgi disruption, surface 'blebbing' and finally disintegration into membranes-bound bodies (Kerr, Wyllie et al. 1972). Maintenance of membrane integrity can be demonstrated by the exclusion of vital dyes such trypan blue and propidium iodide. Necrotic cells are permeable since they cannot maintain the ion gradients required to actively pump out the dyes.

The importance of apoptosis is reflected in the level of redundancy between its multiple effector mechanisms. However, the most completely understood mechanism is that under the control of a family of cysteine proteases known as the caspases. First elucidated in *Caenorhabditis elegans*, activation of the mammalian homologues of

CED-3 are thought to commit cells to the death pathway through a cascade of protein cleavage (including nuclear lamins and cytoskeletal actin), disrupting the cellular repair processes and inducing the morphological changes described above (reviewed in (Nicholson and Thornberry 1997)). In this mechanism, apoptosis is under negative regulatory control by CED-9, which can bind CED-4 thereby preventing caspase activation (Chinnaiyan, O'Rourke et al. 1997; Spector, Desnoyers et al. 1997).

1.5-2 Neutrophil apoptosis:

Early studies showed that aged PMNs, in contrast to fresh PMNs, are recognised by Mϕs (Newman, Henson et al. 1982). It was later shown that PMNs are programmed to undergo constitutive apoptosis, a process sufficient for the specific recognition and ingestion by Mϕs, whilst still intact (Savill, Wyllie et al. 1989). Apoptotic cells are swiftly recognised and engulfed by their neighbours or by specialised phagocytes (Savill, Wyllie et al. 1989; Savill, Smith et al. 1992). Failure or delay in this process allows the apoptotic cells to undergo secondary necrosis, leakage of intracellular contents and increases the risk of tissue damage.

However, the lifespan of immune cells can be influenced by exogenous factors. For example, PMN apoptosis is delayed in response to LPS, complement factor C5a and GCs - a characteristic not shared by close lineage relatives, the eosinophils (Lee, Whyte et al. 1993; Meagher, Cousin et al. 1996). Conversely, pro-inflammatory cytokines such as TNFα accelerate the rate of PMN apoptosis (Takeda, Watanabe et al. 1993).

There is great debate upon the specificity of the aPMN/ phagocyte recognition process, and the extent to which PMNs decide their own fate through the expression of surface markers. Fadok has shown that aPMNs can be characterised by the 'flipping' of phosphatidylserine (PS) residues from the inner leaf of the cell membrane to the cell surface, and that Mϕs recognise aPMNs via putative PS-receptors (Fadok, Voelker et al. 1992; Fadok, Bratton et al. 2000). Furthermore, recent work has also shown that healthy PMNs 'fend off' inquisitive Mϕs through surface expression of CD31, and when apoptotic, a now dysfunctional CD31 on the aPMN surface cannot prevent tethering and

phagocytosis occurring (Brown, Heinisch et al. 2002). Figure 1-5 shows the array of ligands and receptors that are thought to be involved in recognition and phagocytosis.

1.5-3 Phagocytes:

In 1891 the Russian biologist Metchnikoff observed the engulfment of PMNs by Mϕs in a 'pricked' tadpole tail (Metchnikoff 1891). These fundamental observations were extended when the successive events of streptococci ingestion by PMNs, followed by PMN engulfment by Mϕs was observed in an erysipelas (acute cellulitis) model (Metchnikoff 1891). Later, in studies of peritonitis, PMN fate was shown to be that of engulfment by inflammatory Mϕs (Sanui, Yoshida et al. 1982). Only in recent years have the importance of these observations been realised. In 1989 Savill et al demonstrated the non-inflammatory, injury-limiting ingestion of intact aPMNs by Mϕs *in vitro* and *in vivo* (Savill, Wyllie et al. 1989). Subsequently this was found to be dependent on specific recognition signals, including Mϕ vitronectin receptor, thrombospondin, CD 36 and/ or PS receptor systems (Savill, Henson et al. 1989; Fadok, Savill et al. 1992; Savill, Hogg et al. 1992).

Of great importance to the inflammatory process are responses elicited by phagocytes during phagocytosis. The engulfment of pathogenic particles, such as opsonized (Ab-coated) zymosan, induces Mϕ release of thromboxane B2 and other pro-inflammatory cytokines (Meagher, Savill et al. 1992). However, phagocytosis of aPMNs is crucially distinguished from the engulfment of pathogenic particles by the failure of the Mϕ to secrete pro-inflammatory factors upon aPMN ingestion (Meagher, Savill et al. 1992; Stern, Savill et al. 1996). Furthermore, it has been shown that this response is dependent on the specificity of the recognition process rather than the nature of the 'feed' since Mϕs elicit a pro-inflammatory response after ingestion of opsonized aPMNs (Meagher, Savill et al. 1992).

Inflammatory Mϕs secrete pro-inflammatory cytokines when activated by LPS or IL-1β (Trinchieri 1997). However, after phagocytosis of aPMNs, not only do Mϕs fail to secrete pro-inflammatory mediators, but they also become programmed to down-

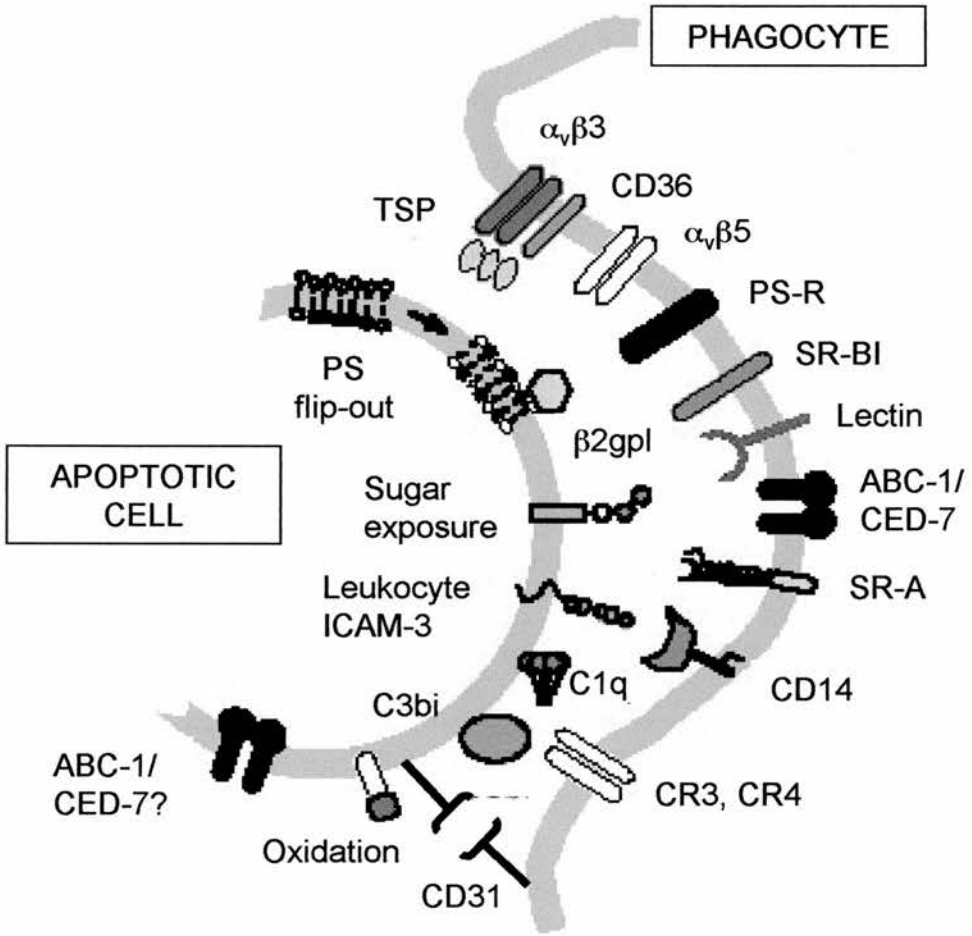


Figure 1-5: Overview of molecules thought to participate in the recognition and phagocytosis of apoptotic cells. Diagrammatic representation of molecules active at apoptotic-cell surfaces and at phagocyte surfaces. Some molecules, TSP, β2gpI and complement components appear to act as molecular bridges between apoptotic cells and phagocytes. For convenience, the phagocyte shown is a 'hybrid' of multiple professional and non-professional phagocytes. Details of receptor–ligand interactions are unknown but it is thought that the interaction persists once the repulsive effects of CD31 are down-regulated by the apoptotic process. ABC1- ATP-binding cassette transporter 1, β2gpI- b(2) glycoprotein I, CR- complement receptor, PS- phosphatidylserine, SR- scavenger receptor, TLR- Toll-like receptor. Diagram adapted from (Gregory 2000; Brown, Heinisch et al. 2002).

regulate Th-1 cytokine release upon subsequent activation (Voll, Herrmann et al. 1997). This appears to be due to the Mϕ release of anti-inflammatory TGFβ (Fadok, Bratton et al. 1998). This is possibly a mechanism for autocrine deactivation in which a 'fed' Mϕ secretes TGFβ, which in turn suppresses TNF-α release, thus shifting towards an anti-inflammatory phenotype, which may be fundamental to successful resolution.

So far my introduction has focused solely on the Mϕ as phagocyte. However phagocytes can be divided into 2 groups, depending on their primary roles. 'Professional phagocytes' are those such as human MD Mϕs, Mϕs of the human liver, alveolar and synovial fluid, murine thioglycollate-elicited peritoneal (TEP) Mϕs and bone marrow-derived (BMD) Mϕs (Savill, Wyllie et al. 1989; Fadok, Savill et al. 1992; Cox, Crossley et al. 1995; Falasca, Bergamini et al. 1996). In contrast, 'semi-professional phagocytes' are cell types with a predominantly different function that can also assume a phagocytic role when required, such as glomerular MC and fibroblasts. They are thought to ingest neighbouring cells undergoing apoptosis in non-inflammatory situations, as well as assist 'professional phagocytes' in instances where they are overwhelmed by the number of aPMNs (Savill, Smith et al. 1992; Hall, Savill et al. 1994).

1.5-4 Mesangial cells:

MC are myofibroblasts that serve a structural role for the glomerulus and provide housekeeping functions for the maintenance of normal renal physiology. Inflammatory disease of the kidney, glomerulonephritis, may resolve but since it is usually presents late it often persists leading to the loss to MC, glomerular scarring and renal failure (Shimizu, Masuda et al. 1996; Sugiyama, Kashihara et al. 1996). MC can assume a potentially damaging inflammatory role in such diseases through the release of enzymes, vasoactive endobiotics, extra cellular matrix components, prostaglandins and cytokines such as TNFα and IL-β (Pfeilschifter, Schalkwijk et al. 1993). Such pro-inflammatory secretion is suppressed by GCs (Nakano, Ohara et al. 1990; Vishwanath, Frey et al. 1993).

In a rat model of glomerulonephritis, Savill and colleagues presented evidence that aPMNs are cleared, *in vitro* and *in vivo*, by both inflammatory Mϕs and MC (Savill, Smith et al. 1992). Furthermore, as well as ingesting aPMNs, MC can phagocytose neighbouring apoptotic MC, thus preventing necrosis and protecting the glomerulus from the release of pro-inflammatory mediators (Baker, Mooney et al. 1994; Shimizu, Kitamura et al. 1995). In accordance with Mϕ studies, the injury limiting potential of MC was further demonstrated when it was shown that the MC-release of pro-inflammatory mediators is down-regulated after the phagocytosis of aPMNs (Hughes, Liu et al. 1997). Curiously however, phagocytosis of aPMNs by cultured human MC requires the presence of serum, whereas the same reaction with Mϕs does not (Savill, Smith et al. 1992).

The untimely apoptosis of MC and the consequences of necrosis have been implicated in the development of renal scarring (Shimizu, Masuda et al. 1996; Sugiyama, Kashihara et al. 1996). Resolution of glomerulonephritis necessitates tissue re-modelling. Recent *in vitro* evidence suggests that Mϕs within inflamed glomeruli can regulate the numbers of MC present by inducing their apoptosis via NO or TNF-α release (Sugiyama, Savill et al. 1999; Duffield, Erwig et al. 2000). Addition of pro-inflammatory cytokines increased MC susceptibility to apoptosis (Duffield, Erwig et al. 2000). This suggests that killing of resident cells mediated by dysfunctional, inappropriately activated Mϕs may be an additional source of tissue injury.

1.5-5 Resolution:

To summarise, recognition, phagocytosis and complete degradation of an apoptotic cell is an extremely efficient form of deletion which has been estimated, *in vivo,* to take less than 1h (Savill 1997). For example, if 1% of cells in a tissue at any one time were apoptotic, a deletion time of 1h would result in the deletion of a quarter of the tissue over 24h. However, from the discussion above, it can be postulated that a failure to clear aPMNs would result in uncontrolled release of toxic contents from secondary necrotic cells and deprive Mϕs of down-regulatory signals. The resulting persistence of both inflammation and macrophage-directed killing of resident cells is associated with

chronic inflammation and autoimmunity (Savill and Fadok 2000; Taylor, Carugati et al. 2000).

1.6 DEFECTS IN CLEARANCE OF APOPTOTIC CELLS

In the previous section I discussed some of the factors that can modulate the rate of PMN apoptosis (refer to section 1.5-2). One can envisage that necrosis and tissue injury would result from any dysregulation in which the rate of apoptosis exceeded the clearance capacity of phagocytosis.

Several causes of perturbed clearance have been identified. Mediators present in the chronic inflammatory environment such as charged molecules and hydrogen ions have been shown to impede phagocytosis of aPMNs (Savill, Henson et al. 1989). Additionally, autoimmunity has been implicated in deficient clearance, and it was recently shown that treatment with anti-neutrophil autoantibodies accelerated both apoptosis and secondary necrosis of activated PMNs, thus reducing the time necessary for safe recognition and phagocytosis (Harper, Ren et al. 2000).

A genetic deficiency of C1q, a complement molecule that bridges between the aPMN and phagocyte, predisposes humans to the kidney pathology systemic lupus erythematosus (Morgan and Walport 1991). C1q-deficient mice display severe glomerular inflammation, and C1q-deficient Mϕs show a clear defect in clearance of administered aPMNs during thioglycollate-elicited (TE) peritonitis (Taylor, Carugati et al. 2000).

1.7 POTENTIATION OF PHAGOCYTE CLEARANCE OF APOPTOTIC CELLS

Many chronic inflammatory conditions are thought to be exacerbated by accumulation and necrosis of granulocytes. It would therefore be of great therapeutic benefit if an increase of phagocytosis could be effected in a defective or overwhelmed phagocyte. Previous reports have shown that phagocytosis of apoptotic cells can either be

augmented by treatment of Mϕs with pro-inflammatory cytokines, or ligation of Mϕ CD-44 (Ren and Savill 1995; Hart, Dougherty et al. 1997). Interestingly, Hart showed that the CD-44 effect was selective to ingestion of aPMNs, with no ingestion of apoptotic lymphocytes (Hart, Dougherty et al. 1997). Clearly, potential therapies based on pro-inflammatory cytokine treatment could produce undesirable side affects, but the possible efficacy of CD-44 treatment remains unexplored.

My research follows from the valuable work of Liu et al, who showed that short-term treatment of Mϕs with synthetic GCs promoted non-inflammatory clearance of aPMNs (Liu, Cousin et al. 1999). A concentration-dependent promotion of phagocytosis was seen with pharmacological doses of GCs in human MD Mϕs as well as murine BMD Mϕs and TEP Mϕs. Furthermore, the GC-mediated effect was not restricted to the ingestion of aPMNs since the ingestion of eosinophils and Jurkat T-cells was also augmented. Nor was it specific to 'professional phagocytes', and it was shown that human and rat MC responded in a similar fashion. In these *in vitro* experiments, the GC effect was mediated via GR (Liu, Cousin et al. 1999).

The specific recognition and phagocytosis of aPMNs by human MD Mϕs, murine Mϕs and MC have previously been attributed to distinct receptor mechanisms (Fadok, Savill et al. 1992; Hughes, Liu et al. 1997; Savill 1997). Therefore the effects of synthetic GCs may represent a novel pan-phagocyte response, and suggest a hitherto unrecognised facet of the anti-inflammatory effects of GC treatment during the resolution of inflammation.

In later studies, MD Mϕs that were differentiated in the presence of GC became programmed to adopt a pro-resolution phenotype in which phagocytic capacity was enhanced via cytoskeletal reorganisation (Giles, Ross et al. 2001). The degree of augmentation of phagocytic response was related to both dose of GC and length of time of exposure (Giles, Ross et al. 2001). Thus, targeting GC action to the blood borne monocytes, or to the phagocyte during an inflammatory response may be attractive for promoting a safe outcome to an inflammatory disease. Clearly the success of this approach would be dependent on minimising the adverse effects of systemic GC

exposure, and therefore administration of GCs locally, and directly to the inflammatory foci would be advantageous.

1.8 AIMS OF THIS STUDY

Many cell types have the ability to amplify GC levels through the re-activation of active GC from inert 11-keto steroids. This is accomplished by the expression and activity of 11β-HSD1. Pharmacological doses of exogenous GCs have been shown to increase Mϕ capacity for aPMNs, whilst endogenous GCs appear to be immunomodulatory. Whilst immunological phenomena such as the induction of apoptosis in thymocytes by endogenous GCs have been described, the extent to which adrenal GCs influence the resolution of inflammation is unexplored. In particular it is intriguing to postulate that a Mϕ may act autonomously and regulate its own exposure to GC through 11β-reductase action.

I set out to test the hypothesis that '**the local modulation of GC action by 11β-HSD1 within the Mϕ augments clearance of aPMNs during inflammation**'.

There were several key aims of the work described in this thesis. For the hypothesis to be valid Mϕs must express 11β-HSD1 and the enzyme would be active in the reductase direction. *Therefore the first aim was to establish 11β-HSD isoform expression and determine enzymatic direction in various types of Mϕs and in MC.*

The hypothesis implies that endogenous GCs would confer an effect *in situ* so therefore it should be possible to augment phagocytosis with physiological concentrations of native GCs. In particular, providing 11β-reductase activity is present, it would be expected that augmentation could be conferred by administration of inert A. *Therefore the second aim was to determine the effect of 11β-reductase action on the phagocytosis of aPMNs by Mϕs in response to native GCs / and to demonstrate the role of 11β-HSD1 through the use of enzyme inhibitors.*

Inflammation is a dynamic process tightly regulated by a plethora of inducers and repressors. It follows that Mϕ 11β-reductase activity should be regulated such that GC is amplified if and when required. *The third aim was to examine the regulation of 11β-HSD1 in Mϕs by cytokines and other pro- and anti-inflammatory mediators.*

GCs are required for the differentiation of some cell lineages, and induction of 11β-HSD1 and its associated transcription factors are known to influence cell maturation. Mϕs differentiate, activate, mature and down-regulate and, if 11β-HSD1 expression is important for Mϕ biology, it is probable that Mϕ function or phagocytic ability would be altered in the absence of 11β-HSD1. *Finally, the fourth aim was to use 11β-HSD1-deficient mice to examine the relevance of 11β-HSD1 biology in in vivo models of inflammation and Mϕ function.*

Chapter 2:

Materials and Methods

2.1 MATERIALS

2.1-1 Tissue culture:

All culture media Phosphate buffered saline (PBS) (without calcium and magnesium) Hank's balanced salt solution (HBSS) (without calcium and magnesium) Supplements-	Invitrogen (Life Technologies), PO Box 35, Free Fountain Drive, Incsinnan Business Park, Paisley, PA4 9RF
Sterile tissue-culture plasticware etc-	Costar, Corning Incorporated, Corning, NY 1481.
Teflon culture pots-	Roland Vetter Laborbedarf, e.K, Herrenbergerstr.5, 72119 Ammerbuch, Ger.
Cell Tracker™ fluorophores (GREEN CMFDA (CM-Green))-	Molecular Probes Europe BV, PoortGebouw, Rijnsburgerweg 10, 2333 AA Leiden, The Netherlands.

2.1-2 Reagents and antibodies:

Unless otherwise stated all reagents were purchased from-	Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH.
Agarose-	BioWhittaker Molecular Applications, Wokingham, Surrey, UK.
Amberlite ion exchange resin-	VWR, Merck House, Poole, Dorset, BH15 1TD.
Antibodies (Rat R PE monoclonal antibodies to mouse F4-80 and GR-1 and the corresponding IgG-2b control)-	Caltag Labs, 1849 Bayshore Blvd. #200, Burlingame, CA 94010.
Autoradiograph film (Kodak Biomax MS)	Sigma-Aldrich (see above)
Brewer's thioglycollate powder-	Difco Laboratories, PO Box 331058 Detroit, MI.
Dextran and Percoll-	Amersham Pharmacia, Biotech UK Limited, Amersham Place, Little Chalfont, Bucks HP7 9NA,UK.
Ethanol-	Hayman Ltd, Witham, Essex, UK
Formamide, Formaldehyde (38%) and other solvents	VWR (see above).
IL-4 (Recombinant human IL-4)	R&D Systems, 19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB
Hybond-H nylon membrane-	Amersham Pharmacia (see above).
Hyper film-	Amersham Pharmacia (see above).
Methanol-	VWR (see above).
Nick columns-	Amersham Pharmacia (see above).

Quantikine ELISA system-	R&D systems (see above).
Radio-isotopes ([1,2,6,7-^{3}H]Corticosterone, specific activity 70.0 Ci/mmol and [α^{32}P]-dCTP, specific activity >3000 Ci/mmol).	Amersham Pharmacia (see above).
RNaseZAP-	Ambion, 2130 Woodward St, Suite 200, Austin, Texas.
Saranwrap	SC Johnson Wax (Dow), Frimley Green Road, Frimley, Surrey, GU16 5AJ
Scintillation proximity assay beads-	Amersham Pharmacia (see above).
Scintillation fluid (Pico-fluor 40)-	Canberra Packard, 14 Station Rd, Pangbourne, Berkshire RG8 7DT.
Sephadex G-50 column-	Amersham Pharmacia (see above).
1X trypsin/EDTA-	0.05% (w/v) trypsin, 0.53mM EDTA, made up in Ca^{2+}, Mg^{2+} free Hank's (HBSS).
TRIzol reagent-	Invitrogen (see above).

2.1-3 Molecular Biology

Deoxynucleotide Triphosphates (dNTPs)-	Promega Corporation, 2800 Woods Hollow Road, Madison, WI.
DNA size markers (100 Base-Pair Ladder)-	Amersham Pharmacia (see above).
Random primed DNA labelling kit-	Roche diagnostics Ltd, Bell Lane, Lewes, East Sussex, BN7 1LG
Reverse Transcription System-	Promega Corporation (see above).
Restriction enzymes-	Promega Corporation (see above).
RNA polymerases-	Promega Corporation (see above).
*Taq*BeadTM HotStart Polymerase-	Promega Corporation (see above).
TLC plates (aluminium sheets, 20x20cm, silica gel 60 F-254)-	VWR (see above).

2.1-4 Equipment:

Cytocentrifuge (Shandon 3)-	Shandon Scientific Ltd, 93-96 Chadwick Road, Astmoor, Runcorn, Cheshire, WA7 1PR.
Eppendorf Centrifuge 5415C(<2ml vol)-	Eppendorf AG, Hamburg, Germany.
FACS Calibur, FACS Vantage-	Becton Dickinson, 21 Between Towns Road, Cowley, Oxford, OX4 3LY.
FujiFilm FLA-2000 phosphorimager and Fuji BAS phosphorimager tritium screen-	Raytek Scientific Ltd, Sheffield, UK. Fuji Photofilm Company Ltd, Tokyo, Japan.
Gene Quant RNA/DNA calculator-	Amersham Pharmacia (see above).
Labofuge 400R Centrifuge (>15ml vol)-	Heraeus, Brentwood, Essex, UK
Scintillation counter-	Wallac 1450 Microbeta Plus liquid scintillation counter, 20 Vincent Ave, Crownhill Business Centre, Crownhill, Milton Keynes,MK8 0AB.
Techne hybridisation oven and hybridisation bottles-	Techne, Jencons-PLS, Cherrycourt Way, Stanbridge Road, Leighton Buzzard, LU7 8UA
Thermal Cycler-	Eppendorf Mastercycler Gradient (see above)

2.1-5 Software:

Flow cytometry analysis (CellQuest)-	Becton Dickinson (see above).
Multicalc-	Wallac (see above)
Phosphorimage analysis (Aida)	Raytek Scientific Ltd, (see above).
Statistical analysis (Excel)	Microsoft Corporation

2.1-6 Preparation of buffers and solutions:

Borate buffer-	8.25g Boric acid, 2.7g NaOH, and 3.5ml HCl (33M) made up to 1L with dH_20, pH to 7.4, then add 0.5% BSA (0.5g/100ml).
Carbenoxolone solution-	Carbenoxolone (Fw=614.7g) was dissolved in 100% EtOH to a stock concentration of 10mM and stored at 4°C.
C buffer-	63g glycerol, 8.77g sodium chloride, 186mg EDTA and 3.03g Trizma base made up to 500ml with dH_20 and pH to 7.7.
CM-Green solution-	1mg Cell Tracker GREEN CMFDA resuspended in 1ml DMSO and stored in 10µl aliquots at –20°C.
DEPC-treated H_20-	0.5L dH_20 with 5 drops diethylpyrocarbonate, left o/n before autoclaving.
De-ionised formamide-	Mix 50ml formamide with 3g Amberlite ion exchange resin for 1h, then filter twice.

DNA polymerase reaction buffer (10X)	500mM KCl, 100mM Tris-HCl pH 9, 1% Triton X-100.
0.5M EDTA (pH 8)	0.8L dH_20 with 186.1g $Na_2EDTA.2H_20$. pH was adjusted to 8.0 with NaOH, and volume made to 1L with dH_20, before autoclaving.
ELISA Abs	Capture Ab- Goat anti-mouse TNFα at 0.8μg/ml in PBS. Detection Ab- biotinylated goat anti-mouse at 300ng/ml in reagent diluent.
ELISA Block buffer:	1% BSA, 5% sucrose in PBS with 0.05% NaN_3.
ELISA Reagent diluent:	1% BSA in PBS, pH 7.2, 0.2μM filtered.
ELISA Substrate solution:	1:1 mixtute of colour reagents A (H_2O_2) and B (tetramethylbenzidine).
ELISA Wash buffer:	0.05% Tween 20 in PBS, pH 7.2.
FACS wash:	2g NaN_3, 1g BSA in 1L 1X PBS.
Griess assay reagents	Sulfanilic acid; 470ml dH_20, 30mls orthophosphoric acid stock and 10g sulphanilamide. N-ethelenediamine solution; 1g N-(1-naphthyl)ethelendiamide dissolved in 500ml dH_20. Both stored at 4°C in the dark.
Loading buffer	250mM Tris-HCl pH 7.5, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 40% glycerol.
10X MOPS buffer	200mM 3-[N-morpholino]propanesulfonic acid, 50mM sodium acetate, 10mM EDTA, pH to 7 then autoclaved.

MPO-specific stain for PMNs	300µl DMB (1.25mg/ml o-dianisidine in dH_2O), 15ml HBBS, 15ml PBS pH 6.2, 4µl (30%) H_2O_2.
Phosphate buffer	0.2M NaH_2PO_4, 0.6M Na_2HPO_4, 5mM EDTA made to 1L in dH_20 and autoclaved.
Reverse transcription buffer (10X)	100mM Tris-HCl pH 9, 500mM KCl, 1% Triton X-100.
20X SSC buffer	175.3g NaCl, 88.23g Na Citrate made to 1L with dH_20 and autoclaved.
Steroids-	Dexamethasone (Dex) (FW=392g), corticosterone (B) (FW=346.5g) and dehydrocorticosterone (A) (FW=344.4g) were dissolved in 100% ETOH to a stock concentration of 10mM and stored at –20°C.
10X TBE buffer	108g Trizma base, 55g boric acid, 20ml 0.5M EDTA, made to 1L in DEPC H_20 and autoclaved.
TE buffer	10mM Tris-HCl pH 8, 1mM EDTA, made to 1L with dH_20, then autoclaved.
Thioglycollate solution (3%)	2.98g of Brewer's thioglycollate powder dissolved in 0.1L dH_20 and autoclaved.

2.1-7 Animals:

Mice homozygous for a targeted disruption of the 11β-HSD1 gene have been described previously (Kotelevtsev, Holmes et al. 1997). The disrupted 11β-HSD1 allele was backcrossed to a C57BL/6 background (8 backcrosses) by Dr. Janice Paterson. Male mice, aged 8-10 weeks, and age-matched C57BL/6 controls (purchased from Harlan Orlac, Bicester, UK) were used in all experiments. Animals were housed under controlled conditions (12h light, 21°C) with unrestricted access to water and standard chow.

2.2 METHODS

2.2-1 Isolation of human leukocytes from fresh blood:

Leukocytes were isolated from fresh human blood by dextran sedimentation of erythrocytes, followed by the fractionation of leukocytes by centrifugation on a discontinuous Percoll gradient as previously described (Savill, Wyllie et al. 1989). This protocol typically yields populations of granulocytes (98% PMNs) and mononuclear cells (25% monocytes/ 75% lymphocytes).

40mls fresh blood was drawn from a healthy volunteer through a 19g butterfly needle and added to 4ml of 3.8% sodium citrate in sterile 50ml polystyrene tubes. 4 such tubes of blood were usually collected and gentle handling procedures were used to minimise PMN activation. The tubes were then centrifuged at 350g for 20min at room temperature (with the centrifuge set on slow deceleration), which separated the erythrocyte fraction from the platelet-rich plasma. This plasma layer was gently aspirated and 10ml was re-calcified with 200μl (5mM) $CaCl_2$ in a sterile glass vial for 1h at 37°C to give platelet-rich plasma-derived serum (PRPDS), to be used later as autologous serum in the aging of PMNs and the differentiation of MD Mϕs.

In order to sediment red cells, 6ml of 6% Dextran (500,000 molecular weight, in normal saline) was added, the total volume made to 50ml with normal saline and then cells were

re-suspended by inversion. After 30min, the leukocyte-enriched upper layer was collected by gentle aspiration into sterile 50ml tubes and centrifuged at 350g for 6min.

Meanwhile, 3 working solutions of Percoll (79%, 68% and 55%) were prepared by diluting a 90% stock (generated by diluting Percoll 9:1 with 10X PBS at 4°C) with 1X PBS. These dilutions are expressed as percentage of the 90% stock. 2.5ml of the 68% mixture was slowly overlaid by pipette on 2.5ml of the 79% mixture in a 15ml polystyrene tube. Next, the pelleted leukocytes from 2 of the 50ml tubes were re-suspended in 2.5ml of the 55% Percoll mixture and overlaid on the 68% layer to form a (3-layer) discontinuous gradient. Thus, the 4 tubes collected would yield 2 sample gradients, which were then centrifuged at 720g for 20min (with no brake).

The mononuclear cells and the granulocytes sedimented to the interfaces between the 79%:68% and 68%:55% layers, respectively. Each cellular layer was carefully removed into fresh 50ml tubes and washed twice by centrifugation at 220g in 1X PBS. The cells were then counted by haemocytometer, before either the granulocytes (PMNs) were aged for 24h or the mononuclear cells were differentiated into MD Mϕs (refer to sections 2.2-3 and 2.2-4.1).

2.2-2 Preparation of cytospins:

In some experiments cells were prepared for light and fluorescent oil microscopy by adherence onto glass slides by cytocentrifugation. Cytospins of PMNs and Mϕs were prepared by cytocentrifuge (Shandon 3) with appropriate chambers and slide clips. 100µl PBS or media containing approximately $0.2X10^6$ cells was loaded into a cytospin chamber and cells were adhered onto a glass slide by centrifugation at 300 rpm for 3min. Slides were then air dried, fixed in methanol (2min), stained with standard eosin (2min) and haematoxylin (2min) and cover-slipped, unless otherwise stated.

2.2-3 Constitutive apoptosis of neutrophils in culture:

Careful isolation of the granulocyte layer from the discontinuous Percoll gradient (section 2.2-1) would typically yield a 98% pure population of PMNs (assessed by cytospin), which were viable (not necrotic) when assessed by the ability to exclude 0.2%

trypan blue solution. PMNs were re-suspended at a density of $4X10^6$/ml in 'complete PMN/ MD Mϕ medium' (Table 2-1), and aged overnight in teflon pots at 37°C, 5%CO_2. Examination by cytospin would typically show that >60% of PMNs had condensed, rounded nuclear chromatin formations and were therefore considered apoptotic (Savill, Wyllie et al. 1989). Figure 2-1 contrasts the morphology of healthy PMNs and aPMNs in an aged culture.

2.2-4 Generation of macrophages:

2.2-4.1 Differentiation of human monocyte-derived macrophages:

Mononuclear cells isolated from the Percoll gradient (refer to section 2.2-1) were re-suspended at a density of $4X10^6$/ml in 'serum-free PMN/ MD Mϕ medium'. The mononuclear cells were initially cultured in 24 well plates for 1h before gentle washing with PBS in order to remove non-adherent lymphocytes. The remaining adherent monocytes were differentiated into MD Mϕs in 'complete PMN/ MD Mϕ medium' over the course of 4d, with a medium change at d2.

2.2-4.2 Differentiation of murine bone marrow-derived macrophages:

Male mice (aged 8-10 weeks) were killed by cervical dislocation and immersed in 75% ethanol. In the sterile conditions of a safety cabinet, the skin was removed from the lower limbs then, using autoclaved surgical instruments, the muscle was dissected from both femurs. After careful separation of the femurs from the pelvis and tibias, the femurs were removed into ethanol-filled universal containers then washed in 1X PBS. The femurs were transferred to a sterile petri dish and cleaned by scraping with a sterile scalpel blade. The proximal and distal ends of each femur were removed just below each joint and, by inserting a 25g needle into the bone cavity, the soft marrow was gently flushed with 5ml 'serum-free Mϕ medium' and collected into a universal container. This was repeated once from the opposite end of the bone.

Cell number was determined using a haemocytometer and yield was normally ~$50X10^6$ cells from each mouse. Cells were cultured in 24 well plates at a density of $0.4X10^6$/ml in 'BMD Mϕ medium'. Medium was replaced every 3d and cells were differentiated by

Growth medium:	Contents:
BMD Mϕ medium	Dulbecco's modification of Eagle's Medium (DMEM)/ Ham's F12 (F12) with 'glutamax', 10% v/v heat inactivated (HI) foetal calf serum (FCS), 10% v/v conditioned L929 supernatant (see below), supplemented with penicillin 500U/ml, streptomycin 500U/ml (final concentration).
Serum-free Mϕ medium	DMEM/F12 with 'glutamax', supplemented with penicillin 500U/ml, streptomycin 500U/ml.
Complete Mϕ medium	DMEM/F12 with 'glutamax', 10% v/v HI FCS, supplemented with penicillin 500U/ml, streptomycin 500U/ml.
Serum-free PMN/ MD Mϕ medium	Iscove's/DMEM, supplemented with penicillin 500U/ml, streptomycin 500U/ml.
Complete PMN/ MD Mϕ medium	Iscove's/DMEM, 10% v/v autologous serum (PRPDS) supplemented with penicillin 500U/ml, streptomycin 500U/ml.

L-929 cells are a murine fibrosarcoma cell line that secretes macrophage colony stimulating factor (M-CSF), essential for the differentiation of BMD Mϕs. In order to limit batch variability in M-CSF concentration, a large volume of L-929 conditioned media was collected and stored at –80°C, to be used when necessary. 15 T162 flasks were each seeded with $5X10^6$ cells in 'complete Mϕ medium' and grown to confluence over 6d. The conditioned medium was removed from the cells and filtered through a 0.5μm pore filter, combined into a general batch and stored in 10ml aliquots.

Table 2-1: Composition of cell culture media.

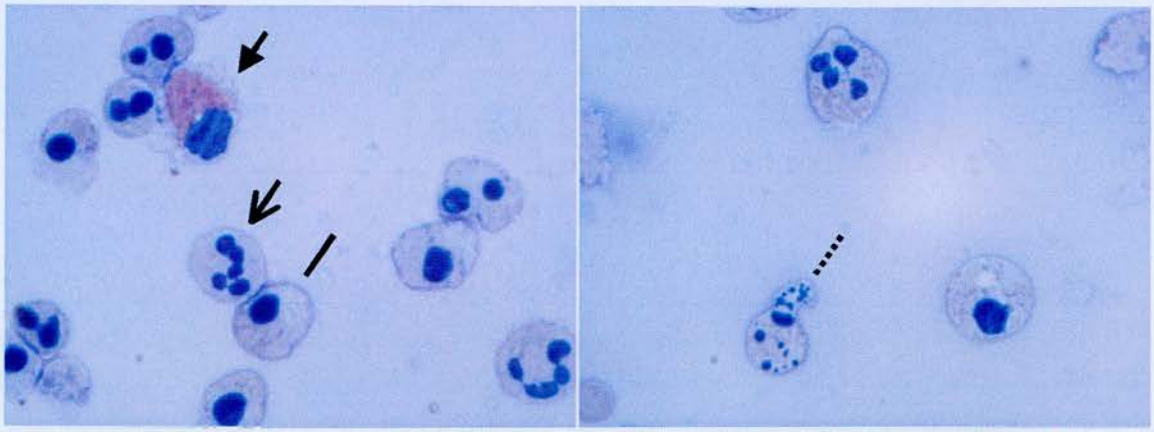


Figure 2-1: Characteristic morphology of aged PMNs. PMNs were isolated from discontinuous Percoll gradients and aged for 24h (as described in section 2.2-3). A healthy polylobular PMN (→), an aPMN (—), a necrotic PMN (⋯) and a contaminating eosinophil (➙) are indicated.

d7. BMD Mϕs were considered as differentiated upon up-regulation of surface F4/80 antigens (by flow cytometry), and attainment of phagocytic competency. This differentiation procedure has previously been validated (Fadok, Savill et al. 1992).

2.2-4.3 Collection of murine peritoneal macrophages:

Inflammatory Mϕs were elicited into the peritoneum by sterile i.p injection of 1ml of 3% thioglycollate solution. TEP Mϕs were then harvested at various time-points. Mice were killed by cervical dislocation (taking care not to rupture the peritoneal cavity) then a subcutaneous incision was made in the abdomen and the skin was separated from the intact peritoneum by blunt dissection. The peritoneal surface was wiped with 75% ethanol and 5ml cold PBS was forcibly injected through a 19g needle. Whilst retaining the needle in position, at least 4ml of peritoneal exudate was withdrawn, transferred into a 15ml Falcon tube and kept on ice. TEP Mϕs were then washed by centrifugation at 4°C and counted using a haemocytometer. At 4d after thioglycollate elicited (TE) peritonitis, a return of 15-20X10^6 cells was typical.

RP Mϕ were collected in a similar fashion from non-injected, healthy mice. It was usual for a healthy mouse to yield approximately 5X10^6 RP Mϕs. In the event that this number was significantly higher, or neutrophilia was observed, the cells were discarded because an un-induced peritonitis was in progress.

2.2-4.4 Selective monocyte depletion:

Access to a murine transgenic model of selective monocyte depletion (on a fvb/nj background) was generously provided by Dr. Jeremy Hughes, CIR. These mice are transgenic for human diphtheria toxin receptor (DTR) placed under the control of the CD11b monocyte promoter. Therefore, when challenged with diphtheria toxin, blood borne monocytes are selectively deleted from the circulation for approximately 48h. PMNs and tissue Mϕs do not appear to be affected and no evidence of tissue damage is observed (Dr. Jeremy Hughes, personal communication). A toxin dose of 25ng/g body weight was given intravenously, 24h before sterile peritonitis was induced.

2.2-5 Establishment of mesangial cell cultures:

The mesangial cells (MC) were a gift from Dr. Jeremy Duffield, CIR, and have been described and characterized previously (Kitamura, Burton et al. 1995; Duffield, Erwig et al. 2000). They were originally derived by clonal selection from single cell preparation of glomeruli from young Sprague-Dawley rats. MC grow clonally in culture for up to 100 passages, but are typically used between the 10^{th} and 15^{th} passage. MC were cultured in 'complete Mϕ medium' and were split every 4d. 24h prior to experiments, MC were removed from flasks by Trypsin/EDTA treatment and re-seeded in 1ml at a density of $0.1X10^6$ cells/ml in 24 well plates. Typically each treatment would be over the course of 24h, and consequently, 48h after seeding, confluency would be reached.

2.2-6 RNA extraction and analysis:

Great care was taken to protect RNA samples from contamination and degradation by nucleases. All glassware and tubes were autoclaved, and all equipment was treated with RNaseZap prior to use. All solutions were made with DEPC H_20 or were used from previously unopened nuclease-free stocks.

2.2-6.1 Extraction of total RNA by TRIzol:

TRIzol reagent enables a single step isolation based on a modification of the Chomczynski and Sacchi method of RNA extraction using guanidium thiocyanate (Chomczynski and Sacchi 1987).

Portions of liver and kidney were dissected onto dry ice then ground to a paste in liquid N_2 by mortar and pestle. The liquid N_2 was allowed to evaporate and, following the manufacturer's protocol, 1ml of TRIzol solution was added to ~75mg of powdered tissue. Alternatively, 1ml of TRIzol was added to $7.5X10^6$ cells harvested from Mϕ or MC monolayers. The cell suspension was mixed thoroughly and either frozen at –80°C for later use, or used immediately. 0.2ml chloroform (per 1ml TRIzol) was added to the suspension, inverted several times, incubated at room temperature for 3min, and then centrifuged at 12,000g at 4°C for 15min to separate the phenol and aqueous phases. The

upper aqueous phase containing RNA was removed (care was taken not to touch the DNA containing interface or the lower protein-containing phenol-choloform phase) and RNA precipitated by the addition of 0.5ml isopropanol with thorough mixing, incubation at room temperature for 10min and centrifugation at 12,000g at 4°C for 10min. The supernatant was removed and the RNA pellet was washed with 1ml 75% ethanol by centrifugation at 7,500g at 4°C for 5min. The pellet was finally allowed to partially air dry (so as not to compromise solubility) and was then dissolved in 20µl nuclease-free water by pipetting and incubation for 10min at 60°C. Samples were stored at -80°C until required.

2.2-6.2 Assessing RNA integrity:

Concentration and purity were assessed by measurement of optical density, using a GeneQuant RNA/DNA calculator. 1µl RNA solution was diluted in 100µl DEPC H_20 and quantified by UV absorbance at wavelengths of 260nM and 280nM. A ratio of >1.6 indicates RNA that is free of protein and not de-purinated. RNA integrity was also checked by electrophoresis of 500ng on a standard 50ml 1% Agarose/ TBE gel containing 0.005% ethidium bromide (EtBr). Visualisation of the gel under UV light showed 2 distinct bands (28S and 18S rRNA) above a larger, more diffuse band of mRNA.

2.2-6.3 Reverse transcription of mRNA to cDNA:

Reverse transcriptase synthesizes single-stranded cDNA from total RNA, and the cDNA product can be used directly in PCR reactions.

Using Promega's Reverse Transcriptase System, a 20µl reaction mixture was prepared containing 50ng/µl total RNA, 5mM $MgCl_2$, 1X Reverse Transcription Buffer, 1mM each dNTP, 1U/µl RNasin (ribonuclease inhibitor), 15U/µl reverse transcriptase and 0.5µg oligo$(dT)_{15}$ primer. Using a 'quick-ramping' thermal cycler the mixture was incubated at 42°C for 30min to allow extension of the primer, then heated at 99°C for 5min to inactivate the reverse transcriptase and finally rapidly cooled to 4°C. Negative

controls containing DEPC H_20 instead of either reverse transcriptase or total RNA were performed in parallel.

2.2-6.4 Detection of 11β-HSD1/2 mRNA by PCR:

Promega's *Taq*Bead Hot Start Polymerase protocol allows the release of Taq polymerase from a paraffin bead at 60°C, thus minimising the risk of amplifying products generated from primer-dimer formation or non-specific priming occurring in the reaction mixture at low temperatures.

The following components were combined in a thin walled 0.5ml PCR reaction tube in DEPC H_20 to a volume of 50µl; 5µl of reverse transcription reaction product, 1X DNA polymerase reaction buffer, 0.2mM of each dNTP, 1.5mM $MgCl_2$, 0.1µM of each primer. Lastly, 1 *Taq*Bead (1.25U/bead) was added. Upon melting, the wax is not enough to seal the reaction, therefore each reaction was additionally sealed with mineral oil. Negative controls containing DEPC H_20 instead of either reverse transcriptase or total RNA were performed in parallel.

The primer sequences were:

11β-HSD1,	5' primer, 5'- AGGATCCAG/AAGCAAACTTGCTTGCA –3'
	3' primer, 5'- AAAGCTTGTCACA/TGGGGCCAGCAAA –3'
11β-HSD2,	5' primer, 5'- GCTGCTGCAGATGGACCTGACCAAGC –3'
	3' primer, 5'- GCTCATGTATTTCATCCACCACTA –3'

The cycle conditions were 95°C for 4min, 62°C for 45s, 72°C for 1min. A total of 35 cycles were carried out followed by a 10min extension at 72°C, before being held at 4°C for retrieval. The 11β-HSD1 amplify a 461bp fragment of mouse, human and rat cDNA and have been described previously (Rajan, Chapman et al. 1995). The 11β-HSD2

primers were a gift from Dr. Roger Brown, MMC. They amplify a 144bp fragment in the C-terminal half of the coding region of mouse, human or rat cDNA.

2.2-6.5 Assessing RT-PCR product:

RT-PCR products were run on a 150ml 1% Agarose/ TBE gel containing 0.005% EtBr. 10μl product was mixed with 1μl loading buffer and electrophoresis was carried out in TBE at 150V for 30min, then the gel was viewed under UV light (wavelength 260nm). 1μg of DNA size markers (100 base-pair ladder) was used in order to confirm sizes of amplified products. 11β-HSD1 and 11β-HSD2 amplification is confirmed by the presence of 461bp and 144bp bands respectively.

2.2-7 Northern blotting

2.2-7.1 RNA electrophoresis :

Total RNA was separated on a 100ml 1.2% agarose/ formaldehyde denaturing gel. 1.2g of agarose was dissolved in 98ml 1X MOPS buffer in a microwave oven. Once cooled to 50°C, 2ml formaldehyde (37% stock) was added. The molten gel was poured into a sealed gel tray with combs in place, allowed to set over 1h, and pre-soaked in an electrophoresis tank filled with 1X MOPS buffer for approximately 30min.

25μg samples of RNA were added to DEPC H_20 to give a volume of 10μl, and then 2.5μl formaldehyde (37%), 2.5μl 10X MOPS buffer and 10μl de-ionised formamide were added and mixed thoroughly. Samples were then heated for 15min at 65°C in order to denature the RNA. On ice, 2μl loading buffer was added to each sample before electrophoresis at 100V for approximately 3-4h, or until the bromophenol blue dye front had advanced 3/4 of way down the gel. The inclusion of 0.005% ethidium bromide to each sample at the loading stage allowed the RNA to be briefly visualised under UV, permitting the integrity of the RNA and the equivalence of loading to be assessed.

2.2-7.2 Capillary transfer to nylon membrane:

To allow capillary transfer of RNA, a piece of nylon membrane, and 3 pieces of filter paper of identical sizes to the gel to be blotted were pre-soaked in 20X SSC. A wick of

similar width to the gel, but double the length was cut from filter paper and placed on an up-turned gel tray so that the ends were immersed in a reservoir of 20X SSC which would later act as the source of transfer buffer.

On completion of electrophoresis the gel was pre-soaked for 20min in 20X SSC and then inverted onto the wick. The nylon membrane was then placed upon the gel, and the rest of the surface of the gel (apart the area containing RNA) was sealed with clingfilm- thereby ensuring that all capillary transfer of fluid would have to pass directly through the portion of the gel that was in contact with the membrane. Bubbles were gently smoothed from the membrane and the 3 layers of filter paper were placed on top followed by a stack (approximately 3 inches) of dry paper towels. A glass plate was placed on top so as evenly distribute weight across the stack and capillary transfer was left to proceed overnight at room temperature. Following transfer, the apparatus was dismantled and the membrane rinsed in 20X SSC. Both membrane and gel were viewed under UV light to ensure complete transfer of RNA. The RNA was cross-linked by UV irradiation of 700,000 Joules and then the membrane was baked at 80°C for 2h.

2.2-7.3 Hybridisation to ^{32}P- labelled 11β-HSD1 cDNA:

The nylon membrane was soaked in 20X SSC for 10min, and then pre-hybridised by placing in a pre-warmed hybridisation bottle containing 12ml phosphate buffer and 6ml 20% SDS at 55°C. Sonicated salmon testis DNA (10mg/ml; 100µl) was denatured at 99°C for 10min, cooled on ice and then added to the hybridisation mixture and pre-hybridisation proceeded at 55°C for 3h.

Meanwhile, rat 11β-HSD1 cDNA was labelled with [α^{32}P]-dCTP using a random primed DNA labelling kit (Boehringer Mannheim). The cDNA encoding rat 11β-HSD1 was a 1.1kb EcoR1 fragment which has previously been described (Agarwal, Monder et al. 1989). 25ng of cDNA , made to 9µl with dH_20 was denatured at 100°C for 10min and immediately placed on ice. To this was added 2µl hexanucleotide primer reaction mix supplied with the kit, 1µl each of dTTP, dATP and dGTP, 5µl [α^{32}P]-dCTP and 1µl (5U) Klenow enzyme before briefly mixing and incubating for 1h at 37°C. After this

time, the reaction was briefly heated to 99°C to inactivate the polymerase and then the labelled cDNA was separated from unincorporated [α^{32}P]-dCTP by passage over a Nick column equilibrated with 3ml TE buffer. The reaction was added to the Nick column and two 400µl volumes of TE buffer were eluted through the column. The first was discarded and the second, (containing ^{32}P-labelled cDNA) was collected. 1µl of this purified probe was added to 1ml of scintillant fluid and counted on a liquid scintillation counter. Typically a 1µl aliquot of purified probe contained 4X10^7 cpm. The probe was then denatured (100°C for 5min) and added to the pre-hybridisation mixture for over-night hybridisation at 55°C.

The hybridisation mixture was disposed of and the membrane was washed to remove non-specific hybridisation. An initial rinse in 1X SSC, 0.1%SDS was followed by 2 room temperature washes in 1X SSC, 0.1%SDS each for 20min. The membrane was then washed in 0.3X SSC, 0.1%SDS for 20min at 55°C. The [α^{32}P] on the membrane was monitored by Geiger-Muller counter, and if deemed too high, the final wash was repeated. Without letting the membrane dry, it was wrapped in Saranwrap and exposed to autoradiographic film for up to 2 weeks at -80°C.

2.2-8 Steroid/ drug treatment of macrophages and mesangial cells

2.2-8.1 Steroid/ carbenoxolone treatment *in vitro*:

Stock steroids and carbenoxolone were diluted in 1xPBS to working concentrations. Control cultures were treated with the appropriate concentration of ethanol (EtOH) vehicle (equivalent to that of the treatment groups). No effects are of EtOH were found at concentrations less than 0.001%. Steroids were added for 24h or 48h, as stated in the text.

2.2-8.2 Collection of pro-inflammatory supernatant:

4h after sterile i.p injection of 1ml of 3% thioglycollate solution, mice were killed by cervical dislocation and their peritoneal cavities were exposured by dissection as described in section 2.2-4.3. A 19g needle was inserted intra-peritoneally and with gentle pressure by hand to either side of the peritoneum, fluid pooled in the cavity

formed by the bevel of the needle. Typically, approximately 1ml of exudate fluid was recovered from each mouse. The exudate was centrifuged at 300g for 6min to remove cell debris and the supernatant collected under sterile conditions. The active constituents of this supernatant were not investigated, however 50µl was added to $1X10^6$ Mϕs cultured in each well of a 24-well plate. Control cells were treated with PBS.

2.2-8.3 Steroid treatment *in vivo*:

0.4 mg/kg of Dex was injected i.p on d2 of TE peritonitis using a 25g needle. This gave a total body concentration of approximately 200nM, assuming a 25g mouse had a total volume of 25ml.

2.2-9 Measurement of 11β-HSD activity

11β-HSD activity is reported as % conversion of 200nM A to B, or 200nM B to A, representative of 11β-reductase activity or 11β-dehydrogenase, respectively.

2.2-9.1 Preparation of [^{3}H] 11-dehydrocorticosterone:

[^{3}H]-A is generated from commercially available [^{3}H]-B by incubation with rat placental homogenate. This provides a rich source of 11β-HSD2, which has exclusive 11β-dehydrogenase activity. A high concentration of NAD ensures that co-factor is not limiting.

240µl of stock [^{3}H]-B (in toluene) was dried under a flow of air and re-suspended in 100µl pure ethanol. Approximately one half of a rat placenta was homogenized in 1ml C buffer and 600µl was added to the re-suspended [^{3}H]-B with 400µl 2mM NAD and 8.9ml C buffer. The mixture was split between two 50ml glass tubes, covered and incubated with gentle shaking at 37°C for 3h. The reaction was stopped and steroids extracted by addition of 2 vol ethyl acetate followed by centrifugation (Labofuge) at 2000rpm for 15min. The upper solvent layer was transferred into a clean 5ml glass bottle, dried under air then re-suspended in 500µl ethanol. 1µl aliquots were analysed by HPLC (by C. Christy, MMC) to ascertain purity (routinely >98%) and by scintillation counter to determine specific activity. Subsequent re-analysis showed that [^{3}H]-A is

stable at –20°C for at least 4 months before any discernable loss of purity through reversion to [^{3}H]-B occured.

2.2-9.2 11β-reductase and 11β-dehydrogenase assays:

11β-HSD reductase and dehydrogenase activity in intact cell monolayers was determined as previously described (Low, Chapman et al. 1994), with minor modifications. Tritiated steroids and 'cold' steroids were added to 1ml culture medium so that the final concentrations were 2nM [^{3}H]-A or -B combined with 200nM unlabeled A or B respectively. Thereby the specific activity was reduced 100-fold, and the total steroid concentration was 202nM. 1ml steroid was then overlaid onto monolayers of 1X10^6 Mϕs or MC in 24-well plates and, in order to measure product formation with time, 200μl samples were taken at various times. A control in which steroid was added to an empty well was included to give background level of conversion and sample purity.

2.2-9.3 Thin layer chromatography:

Steroids were extracted from culture medium in 2 vol ethyl acetate by centrifugation (Eppendorf) at 12,000 rpm for 10min. The organic phase was removed to a glass vial and dried under air. [^{3}H]-steroids were re-suspended in 50μl ethanol (containing a mixture of unlabeled A and B in ethanol, each at 5mg/ml, in order to aid visualisation of product separation under UV light at a later stage). 10μl of the samples was repeatedly spotted onto a thin layer chromatography (TLC) plate until all 50μl had been loaded onto its lane and the plate placed in a solvent tank containing 100ml of chloroform/ ethanol (92:8). After approximately 45min the solvent front had reached the top of the plate and, once dry, the separation of A and B was evident by the ability of the steroids to fluoresce under UV light. Quantitation of conversion was achieved by 5d exposure of the TLC plate to tritium sensitive phosphorimager screen and phosphorimager analysis, using Aida software. 11β-HSD1 activity is calculated as % conversion of 200nM A to B (11β-reductase) or B to A (11β-dehydrogenase). This was achieved by imaging plate IP analysis, during which the intensity (pixelation) of background was subtracted from the

intensities of samples. % conversion was then calculated by comparison with the treatment only control.

2.2-10 Phagocytosis assays

2.2-10.1 *In vitro* phagocytosis assay:

The *in vitro* phagocytosis assay for both Mϕs and MC has been previously described (Savill, Henson et al. 1989; Savill, Wyllie et al. 1989; Savill, Smith et al. 1992). It takes advantage of the fact that myeloperoxidase is present in PMNs but not Mϕs or MC. Using hydrogen peroxide and dimethylbenzidine as substrate, myeloperoxidase (MPO) activity stains PMNs brown and therefore PMN engulfment within translucent Mϕs can be easily scored by inverted light microscopy (Newman, Henson et al. 1982).

Phagocytosis assays were only performed with aged PMN populations that were >60% apoptotic by morphological analysis. Aged PMNs were washed in 1X PBS by centrifugation at 220g, and re-suspended in the appropriate medium at a number approximately 3-4 times that of the Mϕ or MC monolayer. PMN suspensions for Mϕ interactions were in 'serum-free PMN/ MD Mϕ medium', whilst those for MC interactions were in 'complete Mϕ medium' (Savill, Smith et al. 1992).

Mϕ (MD, BMD and TEP Mϕs) and MC monolayers were gently washed with 1X PBS, and 1ml of the aged PMN suspension was overlaid onto the phagocyte monolayer. After incubation for 30min or 2h (Mϕ and MC respectively) at 37°C, 5% CO_2, medium was removed and cells were gently washed by aspiration with cold 1X PBS to remove non-ingested PMNs then fixed with 2% formalin solution for 15min.

After fixing, the cells were washed with 1X PBS and stained with 500µl MPO-specific PMN solution for 30min at 37°C and cells visualised by inverted light microscopy. *In vitro*, PMNs are still largely intact within the Mϕs after these short incubation times, and therefore it was possible to determine both the number of Mϕs which have ingested one or more PMNs and the 'phagocytic index'- a more accurate measure of phagocytic capacity in which the number of aPMNs ingested by 100 Mϕs were scored.

In experiments in which latex beads were used as phagocytic feed, 0.2μm fluorescent beads were suspended in 'serum-free PMN/ MD Mϕ medium' and added to $1X10^6$ Mϕs (at a ratio of 5:1) for 30min. Latex beads are easily observed by either light or fluorescent microscopy.

2.2-10.2 *In vivo* phagocytosis assay:

A fluorescent based assay to measure TEP Mϕ phagocytic capacity *in vivo* was developed (Dr. Graham Thomas, CIR) in which CM-Green labelled PMNs were exogenously administered by i.p injection. CM-Green is a lipophilic thiol group-reactive dye that has been shown to reproducibly label PMNs without compromising the constitutive apoptotic pathway or ability to be ingested (Stuart, Lucas et al. 2002).

Prior to aging, freshly harvested PMNs were suspended at $20X10^6$/ml in 'serum-free PMN/ MD Mϕ medium' containing 5μl of 1mg/ml CM-Green solution. Cells were incubated for 25min to allow labelling, after which time cells were diluted to $4X10^6$ with 'complete PMN/ MD Mϕ medium'. The addition of serum for aging at this stage is also advantageous since serum proteins 'mop up' free CM-Green that has not been taken up by the PMNs and which would otherwise be carried over into the phagocytosis assay. PMNs were then aged as described in section 2.2-3.

CM-Green labelled aged PMNs were washed once in 1X PBS by centrifugation at 220g and re-suspended at $30X10^6$/ml in 1X PBS. Using a 19g needle, $30X10^6$ PMNs (approximately 2:1 ratio to TEP Mϕ, see section 2.2-4.3) were carefully injected i.p into mice at various stages of TE peritonitis. After exactly 10min, mice were killed by cervical dislocation and peritoneal exudate was collected onto ice using a 5ml bolus of PBS through a 25g needle as described in section.

2.2-10.3 Immuno-labelling of macrophages for fluorescent microscopy and flow cytometry analysis:

To inhibit antibody capping, all procedures were carried out at 4°C. $0.5X10^6$ Mϕs or peritoneal cells were washed with 1X PBS by centrifugation at 220g and re-suspended in 200μl FACS wash containing 10% FCS (in order to block non-specific binding) by

gentle agitation in polystyrene tubes. After 15min, anti-F480 PE or rat IgG2b-PE isotype antibody was added at a 1:50 dilution (manufacturer's suggested concentration) and incubated in darkness for 45min. (F4/80 is a Mϕ surface antigen that is commonly used as a marker of differentiation). Unbound antibody was removed by 2 washes with 1X PBS by centrifugation at 300g for 5min.

For fluorescent microscopy, cells were fixed in 2% formalin for 15min and cytospins prepared as described in section 2.2-2. Once air-dried, cover-slips were applied in order to view the cells under oil.

For flow cytometry analysis, cells were re-suspended in 300µl PBS and specific and non-specific fluorescence determined by processing >5000 cells/sample through a FACS Calibur machine, using Cellquest software. It was necessary to include appropriate controls to aid gating. Controls included PMNs alone and TEP Mϕs/ peritoneal cells alone. For flow cytometry-based experiments in which surface GR-1 expression was to be detected, anti-GR-1 PE antibody was used under identical conditions as anti-F4/80 PE.

2.2-10.4 Quantifying phagocytosis by flow cytometry:

Due to the time-consuming nature of scoring MPO-stained cells by eye, a flow cytometry based protocol was developed to quantify phagocytosis levels. PMNs were stained with CM-Green prior to interaction with Mϕs. Once harvested, the cell population was stained with anti-F4/80 (refer to in section 2.2-10.3) and taken to FACS Calibur. Controls included PMNs alone and Mϕs alone, and appropriate rat-anti mouse isotype. F4/80 labelled Mϕs and CM-Green-labelled PMNs are gated in FL2 and FL1, respectively. The comparative shift of a population into the red/green quadrant is representative of interaction between a Mϕ and an aged PMN, and was analysed using CellQuest software. The geometric mean fluorescence of cells positive to isotype control was used in analysis. Such are the fluid pressures exerted upon the cells during flow cytometry that this interaction is most probably that of ingestion, rather than binding.

2.2-11 Analysis of peritoneal supernatant

2.2-11.1 Measurement of TNFα levels:

TNFα levels were measured by ELISA. Following collection of TEP Mϕs, the supernatant from the lavage was collected by centrifugation at 4°C, 220g for 15min, and stored at –80°C until use. TNFα levels were detected by Quantikine ELISA system carried out according to the manufacturer's instructions. 96 well plates were coated with capture antibody (100µl per well), sealed and incubated o/n on a shaker at room temperature. Each well was aspirated and carefully washed 3 times with wash buffer. Each well was blocked with 300µl blocking buffer for 2h, and washed as before. Standards (1:2 dilution of 0-1000pg/ml) were prepared with recombinant mouse TNFα diluted in reagent diluent. 100µl of either standards or samples were added per well, sealed and incubated for 2h at room temperature. The plates were washed as before and 100µl Streptavidin-HRP (1:200 dilution of manufacturer's stock in reagent dilutent) was added to each well and the plate was incubated in darkness for 20min, before the washing procedure was repeated. 100µl substrate solution was added to each well and the plate was incubated in darkness for 20min before addition of 50µl stop solution (2N H_2SO_4). Optical densities were determined using a microplate reader set to 450nM, and TNFα concentrations were determined by comparison to the standard curve.

2.2-11.2 Measurement of NO levels:

NO activity was measured indirectly by Griess assay which detects nitrite, the major stable product of NO generation.

Equal volumes of Griess reagents (sulfanilic acid and N-ethelenediamine) were mixed together immediately prior to the assay. 140µl peritoneal supernatant was mixed with an equal volume of Griess reagents in a 96-well plate and absorbance measured after 15min at 540 nm. A standard curve was generated using a serial dilution of sodium nitrite (0-100µM), mixed with equal volumes of Griess reagent and absorbance measured as above. Nitrite concentration in the supernatants was calculated as µM concentration by comparison to the standard curve.

2.2-11.3 Measurement of corticosterone levels:

B levels were measured by radioimmunoassay, in which unlabelled sample B and [^{3}H]-B compete for anti-B Ab. The concentration of B in each sample was determined from the standard curve. However, this method is not a quantitative method for determining free B levels since B is dissociated from CBG. It therefore measures total B concentration.

Samples of peritoneal fluid were diluted 1:5 in borate buffer and heated at 65°C for 30min to denature CBG and allow dissociation of B. A series of B standards were prepared (0-320nM) to allow production of a standard curve. Samples and standards were incubated in duplicate in flexible 96-well plates with a mixture of [^{3}H]-B (10,000cpm added per sample) and B antibody (1 in 10,000 dilution of rabbit anti-rat B antiserum in borate buffer; kindly provided by Dr. Chris Kenyon, MMC) in borate buffer in a total volume of 70µl for 1h at 37°C. Anti-rabbit scintillation proximity assay beads were then added to each sample and the plates sealed and incubated overnight at 37°C. The beads bind to the primary antibody and, if the primary antibody is bound to [^{3}H]-B, the beads cause scintillation of the radioactive signal, detectable by β-scintillation counter using Multicalc software. The inter- and intra-assay coefficients of variation were <10%, and the detection limit was 0.15µg/dl.

2.3 STATISTICAL ANALYSIS

Data are expressed as Mean of values +/- Standard Error of Mean (SEM) as noted in the figure legends. Single or low repetition experiments are described in the text and where appropriate Mean of values +/- data range is shown.

Analysis was performed using Microsoft Excel software. Significance between 2 variables was determined using the student's t-test and, where appropriate, significance between multiple variables was determined by ANOVA. For data analysis, p was set to <0.05 or <0.001, as illustrated in the text by * for $p<0.05$ or ** for $p<0.001$ respectively.

Chapter 3:

11β-HSD1 is expressed in macrophages and mesangial cells, and is active as a reductase

3.1 INTRODUCTION

Recent studies demonstrate a role for Dex in promoting the safe clearance of aPMNs by Mϕ and MC (Liu, Cousin et al. 1999). In these *in vitro* studies, GC was acting via GR; since the effects were abrogated by pre-treatment with the GR antagonist RU38486 (reviewed in Chapter 1). There is considerable evidence that the association between endogenous GC and GR can be profoundly modulated by pre-receptor metabolism of GC by 11β-HSDs (Seckl and Chapman 1997). Of the 2 11β-HSD isoenzymes characterised; 11β-HSD2 exhibits exclusively dehydrogenase activity both *in vitro* and *in vivo*, and 11β-HSD1 is a reductase in most intact cells *in vitro* (and amplifies GC action in specific tissues) (Kotelevtsev, Holmes et al. 1997).

There are only limited reports of 11β-HSD expression in the immune system. 11β-dehydrogenase activity has been detected in stromal cells of the lymphoid organs and, although unproven, the activity is presumed to be that of 11β-HSD2 (Hennebold, Ryu et al. 1996). Indeed, inhibition of this activity was reported to switch cytokine production by activated T cells from a Th1 to Th2 profile. By drawing an analogy with adipocyte differentiation (refer to section 1.4-3), it is possible that local GC action in immune cells could be modified in a cell-specific or stage-specific fashion by the action of 11β-HSDs.

Mϕs and MC are GC-sensitive, and may therefore exhibit 11β-HSD action. The first aim of this work was to establish which, if either, iso-enzyme was expressed and the direction of its activity - thereby establishing a potential for local GC modulation.

3.2 RESULTS

3.2-1 11β-HSD1, but not 11β-HSD2, is expressed in macrophages and mesangial cells

To determine whether 11β-HSD1 and/ or 11β-HSD2 are expressed by phagocytes, RT-PCR was carried out on 1μg RNA isolated from a range of human and murine Mϕs, as well as rat MC (Table 3.1).

Cell type	*Species*
Bone Marrow-derived Macrophages (BMD Mϕs)	C57 BL/6 mouse
Resident Peritoneal Macrophages (RP Mϕs)	C57 BL/6 mouse
Thioglycollate-elicited Peritoneal Macrophages (TEP Mϕs)	C57 BL/6 mouse
11β-HSD1-deficient BMD Mϕs	C57 BL/6 mouse
Monocytes	Human
Monocyte-derived Macrophages (MD Mϕs)	Human
Cultured Mesangial Cells (MC)	Rat

Table 3.1 Range of GC-responsive phagocytes chosen for study.

RNA from liver (which expresses 11β-HSD1) and kidney (which expresses both isoenzymes) were used as positive controls. Appropriate negative controls were included (section 2.2-6.4). Note that the 11β-HSD1/2 primers utilised are not species-specific.

A 461bp RT-PCR product of identical size to the hepatic 11β-HSD1 RT-PCR product (Rajan, Chapman et al. 1995) was synthesized from RNA from murine BMD Mϕs, RP Mϕs and TEP Mϕs. In contrast, no 11β-HSD2 RT-PCR product was detected in murine Mϕ samples, whilst being clearly present in kidney RNA, giving rise to a 144 bp product (Fig.3-1A). No 11β-HSD1 or 11β-HSD2 RT-PCR products were detected in 11β-HSD1-deficient BMD Mϕ samples, confirming the specificity of the reaction (Fig.3-1B).

Interestingly no 11β-HSD1 RT-PCR product was generated by RNA from human monocytes, but was detected in MD Mϕ RNA taken at d2 and d4 of differentiation (Fig.3-1C). Again, no 11β-HSD2 RT-PCR product was detected in these samples.

Similarly, rat MC expressed 11β-HSD1 but not 11β-HSD2 mRNA (Fig.3-1C).

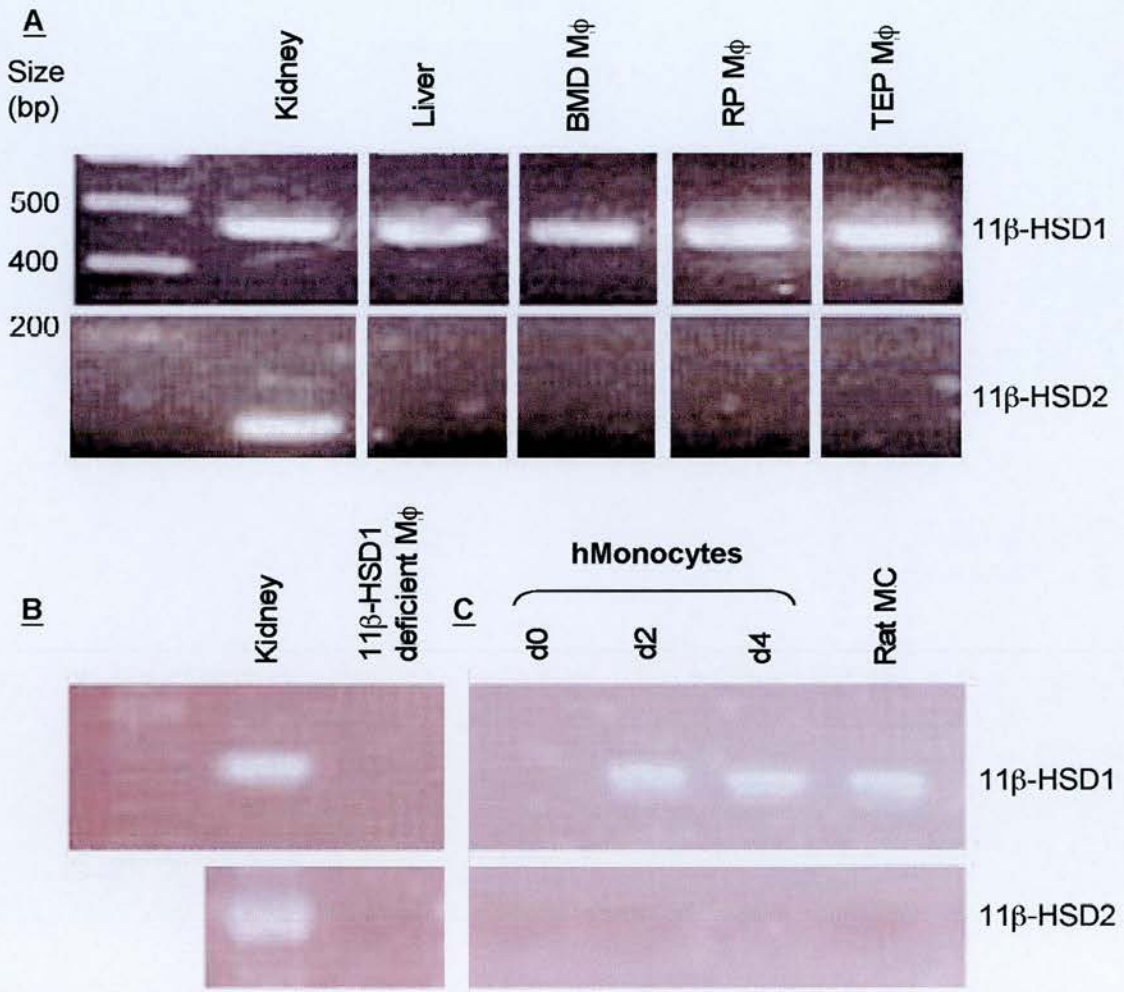


Figure 3-1: 11β-HSD mRNA is expressed in cells of Mφ lineage. A, RT-PCR showing that mRNA for 11β-HSD1 was detected in murine BMD Mφs, RP Mφs, and TEP Mφs (harvested at d3 of TE peritonitis). B, mRNA for 11β-HSD1 was not detected in either BMD Mφs from 11β-HSD1-deficient mice or, human monocytes freshly isolated from peripheral blood (d0) (C). C, both human peripheral blood monocytes (on d2 and d4 of culture) and rat MC expressed 11β-HSD1. 11β-HSD2 mRNA was not detected in any of the cell types studied. Kidney and liver were used as positive controls for 11βHSD-2/1 respectively (kidney expresses both). 1 μg RNA yielded 11β-HSD1 or 11β-HSD2 RT-PCR products of 461 or 144 bp respectively. Images are of single gels, and are representative images of RT-PCR reactions carried out on at least 3 different mRNA samples of each cell type.

3.2-2 Macrophage and mesangial cell 11β-HSD1 mRNA is detected by Northern blot analysis

RT-PCR is a very sensitive method of analysis, detecting even very low levels of expression. Northern blot analysis provides a more quantitative assessment of RNA levels. The expression of 11β-HSD1 mRNA in mouse Mϕs and rat MC was examined by analysis of 25μg total RNA, separated on a denaturing agarose gel. Hybridisation to radiolabelled rat 11β-HSD1 cDNA (91% homology to murine 11β-HSD1 (Rajan, Chapman et al. 1995) identified a single 11β-HSD1 mRNA transcript of 1.4 kb in differentiated BMD Mϕs, TEP Mϕs and rat MC, albeit at lower levels than in liver and kidney (the loading of liver RNA was less than intended due to error) (Fig.3-2). The size of the sample 11β-HSD1 mRNA transcript was verified by comparison to known and well established 11β-HSD1 mRNA transcripts in liver and kidney. Rajan et al used rat 11β-HSD1 cDNA probe to detect murine 11β-HSD1 mRNA by Northern blot (Rajan, Chapman et al. 1995). Very small amounts, if any, 11β-HSD1 mRNA was detected in fresh bone marrow, blood, RP Mϕ or 11β-HSD1-deficient BMD Mϕ samples, despite the loading of approximately equivalent amounts of total RNA. No 11β-HSD2 mRNA was detected in any of the samples, consistent with the negative RT-PCR results (data not shown).

3.2-3 11β-HSD1 functions exclusively as a reductase in intact macrophages and mesangial cells

With very few exceptions, 11β-HSD1 functions in the reductase direction only, although it is bi-directional in homogenates (reviewed in section 1.3).

To determine whether the 11β-HSD1 mRNA present in murine and human Mϕs, and rat MC encodes an enzyme with dehydrogenase or reductase activity, both 11β-dehydrogenase and 11β-reductase assays were carried out on intact cells.

All WT murine Mϕs displayed 11β-reductase activity (Fig.3-3). Similarly, 11β-reductase activity was detected in murine WBC and rat MC, but not in 11β-HSD1-

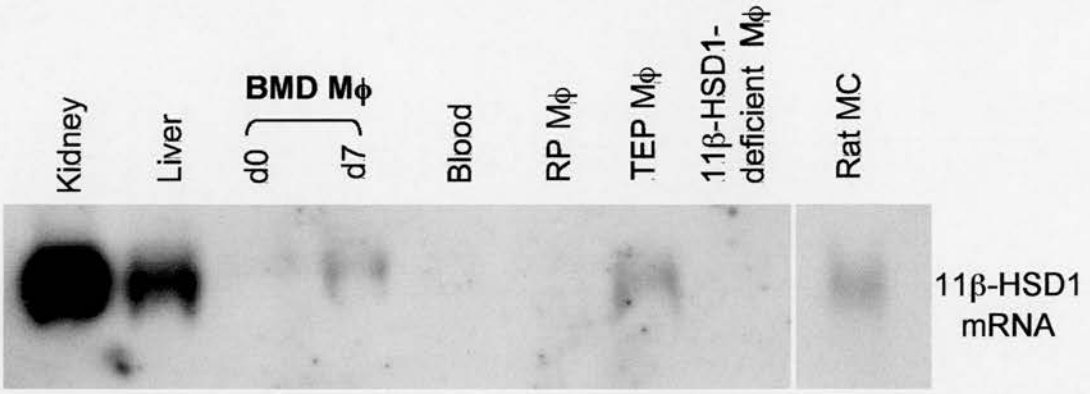


Figure 3-2: Murine Mϕ 11β-HSD1 mRNA is detectable by Northern blot analysis. 11β-HSD1 mRNA was detected in BMD Mϕs cultured for 7d (d7), TEP Mϕs harvested at d3 of TE peritonitis and rat MC. Little or no 11β-HSD1 mRNA was detected in bone marrow cells (d0), fresh blood collected by cardiac puncture, RP Mϕs and BMD Mϕs from 11β-HSD1-deficient mice. Inclusion of EtBr during electrophoresis allowed equivalence of loading to be confirmed under UV light (data not shown). 25μg of each sample was analysed, whereas 40μg of kidney and liver were included as positive controls. Blot exposed to film for 12d.

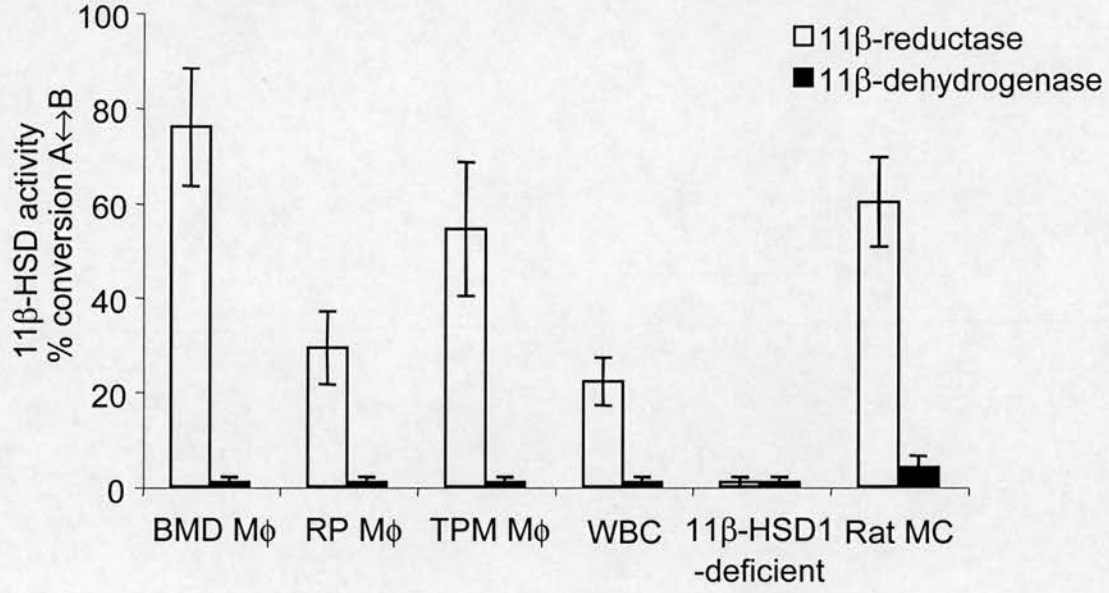


Figure 3-3: Murine Mϕ 11β–HSD1 functions exclusively as a reductase in intact cells. 11βHSD-1 activity is reported as % conversion of 200nM A to B (11β-reductase) or B to A (11β-dehydrogenase) by 10^6 cells over 24h. BMD Mϕs (cultured for 7d), TEP Mϕs harvested at d3 of TE peritonitis and rat MC exhibited significant and exclusive 11β-reductase activity, whereas RP Mϕs and white cells (isolated by red cell lysis from fresh peripheral blood drawn by cardiac puncture) (WBC) exhibited a moderate reductase activity. No 11β-reductase activity was detected in BMD Mϕs from 11β-HSD1-deficient mice (KO). No samples had a 11β-dehydrogenase activity above background level. Values are Mean +/- SEM of 4 separate experiments carried out in duplicate.

deficient Mϕs (Fig.3-3). 11β-reductase activity levels detected in cultures of RP Mϕs and WBC appeared lower than in BMD and TEP Mϕs (Fig.3-3). Mϕs retained the ability to exclude trypan blue exclusion after completion of bioassays, suggesting that the comparative levels measured were representative of inherent Mϕ activity differences rather than unequal loss of cell viability over 24h.

In contrast, 11β-dehydrogenase activity was undetectable in all intact cell types studied (Fig.3-3). The marginal 11β-dehydrogenase activity observed in rat MC culture medium was most probably due to the low percentage of cell death and lysis associated with highly proliferative cell cultures, since it was not present in cultures seeded at low densities (data not shown).

In accordance with the 11β-HSD1 RT-PCR results, no 11β-reductase activity was detected in human monocytes. However, activity was induced by d2 of *in vitro* differentiation (Fig.3-4).

In contrast to murine WBC, no 11β-reductase activity was detected in either human whole blood, or isolated PMNs (Fig.3-4). No 11β-dehydrogenase activity was detected in any human blood cell or MD Mϕ culture (Fig.3-4).

3.3 DISCUSSION

This study shows that 2 classes of phagocytes (Mϕs and MC) express 11β-HSD1, and not 11β-HSD2. 11β-HSD1 transcripts were detectable by RT-PCR and, in some cases, by Northern blotting. Significantly, phagocyte 11β-HSD1 acted exclusively in the 11β-reductase direction in intact cells and reactivates B from inert A.

11β-dehydrogenase activity was not detected - consistent with a failure to detect 11β-HSD2 by RT-PCR.

Whilst the same is true for human MD Mϕs, monocytes did not express 11β-HSD1, and expression and activity were not detected until the d2 of differentiation. Indeed no 11β-

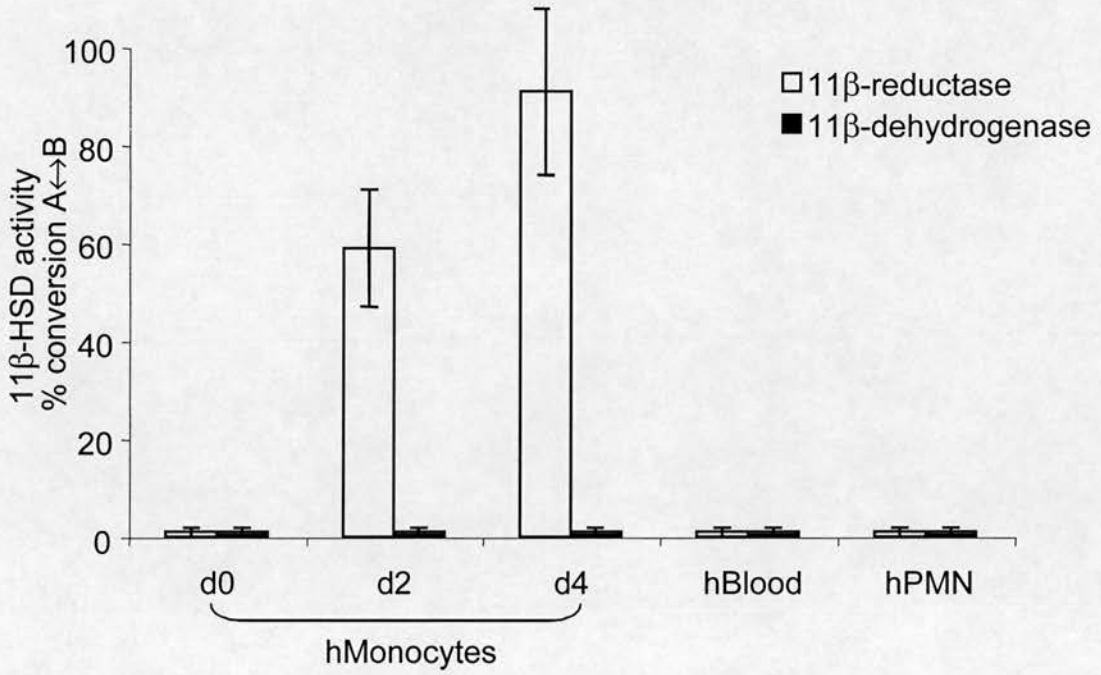


Figure 3-4: Human MD Mϕ 11β–HSD1 functions exclusively as a reductase in intact cells. 11βHSD-1 activity is reported as % conversion of 200nM A to B (11β-reductase) or B to A (11β-dehydrogenase) by 10^6 cells over 24h. Reductase activity was not exhibited by monocytes (d0) but was present by d2 of *in vitro* differentiation. Reductase activity was also undetectable in either fresh peripheral blood or freshly isolated PMNs. No dehydrogenase activity above background level was detected in any sample. Values are Mean +/- SEM of 4 separate experiments carried out in duplicate.

reductase activity was detected in human blood-borne leukocytes. It is likely therefore that, in culture, 11β-HSD1 expression is induced upon differentiation.

Whilst murine 11β-HSD1 could be detected by Northern blot, inconsistency in detection rate necessitated large amounts of RNA to be used, in contrast to liver and kidney where 11β-HSD1 is abundant. Therefore, activity was routinely measured by bioassay as this was reliable, sensitive and provided data on protein, rather than mRNA. Nevertheless, RNA analysis was important in establishing that the enzyme activity was due to 11β-HSD1.

A difference between the 11β-reductase activity in cultures of murine and human blood leukocytes was observed. The activity detected in murine cells was unlikely to be due to sample collection and handling since appropriate sterility and culture practices were observed. It will be of interest to determine if this species difference is indicative of a small mammal's higher metabolic rate and response to stress, or more specifically the C57BL/6 strain's predisposition to a Th1 slanted immune response (Mills, Kincaid et al. 2000). Moreover, it may reflect the animal's health status, or age. Indeed, there is evidence that chronic stress can up-regulate 11β-HSD1 expression in some tissues (Low, Moisan et al. 1994), and indirect evidence that age-associated increases in plasma B may be the result of increased 11β-HSD1 activity (Hennebold, Ryu et al. 1996).

In any case, evidence from this chapter suggests that monocytes and other blood-borne leukocytes have low 11β-HSD1 expression, if any at all. If so, it is of interest that whilst both monocytes and Mϕs are exquisitely sensitive to GC, Mϕs may have a greater potential to amplify GC action through 11β-HSD1 conversion of A to B. Few studies have addressed tissue levels of either A or B, but there is evidence that in areas such as the brain, a significant proportion of the available B is generated from A, locally in the tissue rather than diffusing directly from plasma (Yau, Noble et al. 2001). Monocytes have been shown to strongly express GR (Miller, Spencer et al. 1998). Therefore whilst in circulation they may be entirely under the influence of adrenal-synthesised B, and the differentiation process, if triggered by an inflammatory response may give the

inflammatory TEP Mϕ a level of autonomy from the HPA axis (through the induction of 11β-HSD1) which could be controlled by factors at the inflammatory foci.

The generation of oxygen radicals by NADP(H) oxidase action is a component of the Mϕ's innate response against invading pathogens (Vazquez-Torres and Fang 2001). However, not only is NADP(H) the cellular co-factor which drives 11β-HSD1 reductase direction but it has been shown to be induced in leukocytes taken from inflammatory exudates (Karlsson, Follin et al. 1998).

Thus far the data is consistent with that published by others during my studies. Thieringer et al, studying the presence and induction of 11β-HSD1 during human monocyte differentiation observed a similar phenomenon (Thieringer, Le Grand et al. 2001). Furthermore they reported that 11β-HSD1 expression was induced upon exposure to the anti-inflammatory cytokines IL-4 and IL-13 (Thieringer, Le Grand et al. 2001). Induction was shown to occur with differentiation of human monocytes on plastic or Teflon, demonstrating that 11β-HSD1 induction was not solely a response to plating. As yet it is unknown to what extent the timing of induction *in vitro* is determined by components of the autologous serum, without which differentiation does not occur (Musson and Henson 1979; Newman, Musson et al. 1980).

Rat MC express 11β-HSD1 but not 11β-HSD2 mRNA, whereas whole kidney expresses both. This is consistent with previous studies in which 11β-HSD1 expression was localised to the mesangium and 11β-HSD2 expression was high in MR-rich distal nephron (Krozowski, Albiston et al. 1995; Krozowski, MaGuire et al. 1995; Escher, Galli et al. 1997). As yet, there is no evidence whether MC 11β-HSD1 expression is only induced in culture, or whether it is omnipresent in normal renal physiology. Primary MC are, like Mϕs, GC-responsive (Liu, Cousin et al. 1999) and this therefore may necessitate a constitutive expression of the enzyme to ensure sufficient supply of B. Escher et al have shown that MC 11β-HSD1 expression is up-regulated by cytokine exposure, although in contrast to Thieringer's work, by pro-inflammatory cytokines TNFα and IL-1β (Escher, Galli et al. 1997).

It is attractive to speculate on the reasons why such GC-responsive regulators of the inflammatory response as Mϕs and MCs require a mechanism for GC-amplification, particularly since cells sharing the same precursor origins such as PMNs do not appear to have the mechanism. GCs are immunosuppressive and are thought to mediate a protective shift from a Th1 to a Th2 profile in Mϕs in order to prevent an over-reaction to the immune challenge. In addition, recent evidence shows that they also increase Mϕ and MC capacity to ingest apoptotic cells - a key process in determining inflammatory outcome (Liu, Cousin et al. 1999).

These data show that Mϕs demonstrate potential for the amplification of GC and therefore such action may contribute to the clearance of apoptotic cells. Furthermore this potential is acquired upon differentiation from monocytes, implying a selective role within the peripheral tissues rather than the circulation. In the Chapter 4, the functional consequences of these findings are explored.

3.4 SUMMARY

- Murine bone marrow-derived Mϕs, resident peritoneal Mϕs, and thioglycollate-elicited Mϕs, rat mesangial cells and human monocyte-derived Mϕs exclusively expressed 11β-HSD1, not 11β-HSD2 mRNA. Furthermore, the encoded 11β-HSD1 was active solely in the 11β-reductase direction and therefore should be capable of performing a GC amplifying role.
- Human monocytes did not express 11β-HSD1 mRNA. However, 11β-HSD1 mRNA and activity was detectable at d2 of differentiation.
- Fundamental differences between human and murine blood cells were found. No 11β-reductase activity was detected by human blood cells, whereas murine white blood cells displayed significant 11β-reductase activity over 24h.
- 11β-HSD1-deficient Mϕs did not express 11β-HSD1 mRNA, and consequently 11β-reductase activity was not detected. This demonstrated the specificity of the

RT-PCR and the Northern blotting. Furthermore, this data confirmed that the only enzyme capable of 11β-reductase action in murine Mϕs is 11β-HSD1, and demonstrated its validity for use as a model to investigate the role of 11β-HSD1 in the Mϕ.

Chapter 4:

Consequences of 11β-HSD1 activity *in vitro*

4.1 INTRODUCTION

The capacity of synthetic GCs to promote the phagocytosis of apoptotic cells by Mϕs and MC has recently been reported (reviewed in section 1.7) (Liu, Cousin et al. 1999). Given the expression of 11β-HSD1 reductase activity in Mϕs and MC, there might be a functional consequence in terms of phagocytic function. To determine the potential for a role of 11β-HSD1 in this process, it was investigated whether the action of 11β-HSD1 permitted otherwise inert A to exert the same phagocytic effect as synthetic GCs.

Ultimately the role of 11β-HSD1 in the Mϕ should be tested *in vivo* by utilising 11β-HSD1-deficient mice. During the course of this work 11β-HSD1-deficient mice (Kotelevtsev, Holmes et al. 1997) were bred from a MF1/129 background onto C57BL/6, and were later to become the model of choice to determine the *in vivo* relevance of Mϕ 11β-HSD1. However, much preliminary work was undertaken in this chapter to, firstly determine the GC-responsiveness of Mϕs from a C57BL/6 background and, secondly, to establish the potential of 11β-HSD1 to influence GC action in Mϕ.

Liu et al described phagocytic effects of GCs upon murine Mϕs from a BALB/C background. Mϕs from BALB/c and C57BL/6 mice are considered to display Th-1 and Th-2 slanted immune responses respectively, which can result in fundamentally different inflammatory responses to the same stimuli (Mills, Kincaid et al. 2000). This fact, and the well documented ability of GCs to influence an immune cell's cytokine repertoire, made it imperative to validate Liu's observations on Mϕs from the C57BL/6 mouse. In addition, it was by no means certain that physiological GCs would elicit an identical response to dexamethasone (Dex), since the nature of the synthetic steroid is to achieve increased receptor avidity and longer 1/2 life due to an additional halogen group at its 9-alpha position. It was therefore of interest to discover whether these phagocytic effects extended to the physiological adrenal-GC, B and if so, whether physiological concentrations of B were effective.

Next the question of 11β-reductase relevance was addressed. Would the re-activation of B from inert A deliver the Mϕ with a sufficient concentrations of GC to mediate

phagocytic augmentation? If so, could specificity and manipulation be demonstrated by abolishing the effect with the use of 11β-HSD inhibitors?

From the absence of 11β-HSD expression in human monocytes, it is predicted that monocytes and MD Mϕs ought to have differing responses to A. Is the expression of 11β-HSD1 simply a consequence of differentiation or more intriguingly, a pre-requisite for the phagocytosis of apoptotic cells?

Therefore, a series of *in vitro* experiments were devised to (i) validate the Dex effect on C57BL/6 Mϕs, (ii) investigate the efficacy of physiological steroids and (iii) the consequences of 11β-HSD inhibition on phagocytosis of aPMNs by Mϕs and MC. These studies were then extended to investigate whether a correlation existed between 11β-HSD1 induction and phagocytosis.

4.2 RESULTS

4.2-1 Dexamethasone augments the phagocytosis of apoptotic neutrophils by C57BL/6 macrophages

The effect of Dex on the phagocytic capacity of C57BL/6 Mϕ for aPMNs was determined *in vitro* and *in vivo*. *In vitro*, BMD Mϕs were treated with 200nM Dex after 6d in culture (24h prior to phagocytosis assay) and aged PMNs were overlaid (at a ratio of 4:1) 24h later (refer to section 2.2-10). Similarly, TEP Mϕs harvested 3d after onset of TE peritonitis were adhered to plastic and treated with 200nM Dex for 24h before being overlaid with aged PMNs. In both cases, after an interaction time of 30 min, non-ingested PMNs were removed and Mϕ phagocytosis of aPMNs scored (refer to section 2.2-10.1). Figures 4.1A and 4.2 show that Dex treatment significantly augmented the capacity for phagocytic uptake of aPMNs by BMD Mϕs and TEP Mϕs *in vitro*, consistent to previous reports (Liu, Cousin et al. 1999). Not only did Dex treatment increase the percentage of Mϕs that phagocytose aPMNs but the number of aPMNs ingested by an individual phagocytic Mϕ was also augmented (Fig. 4.2B).

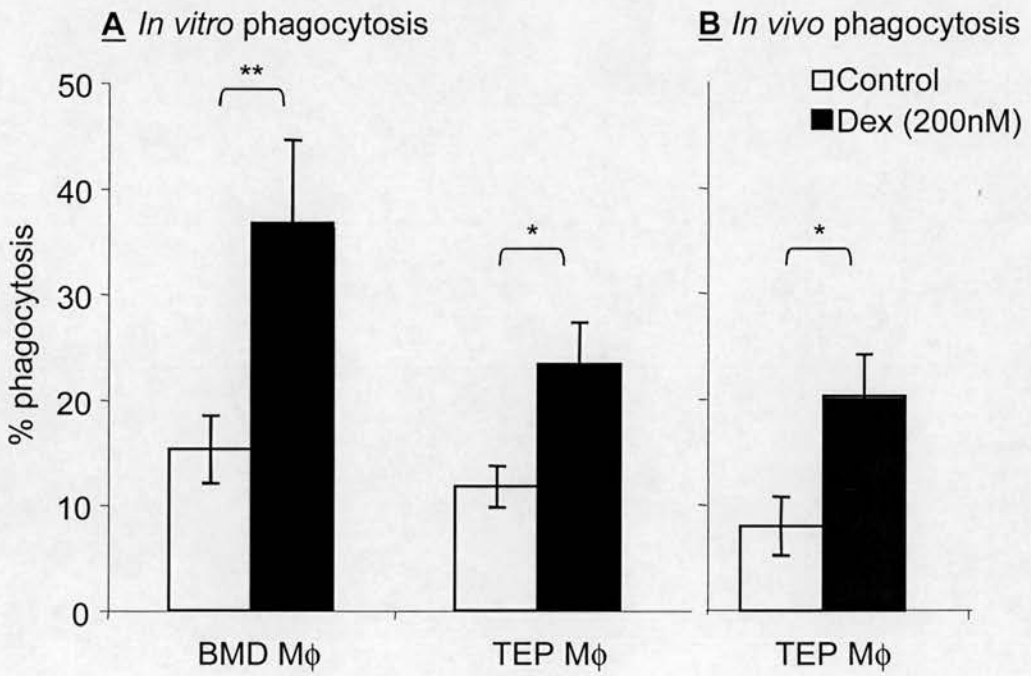
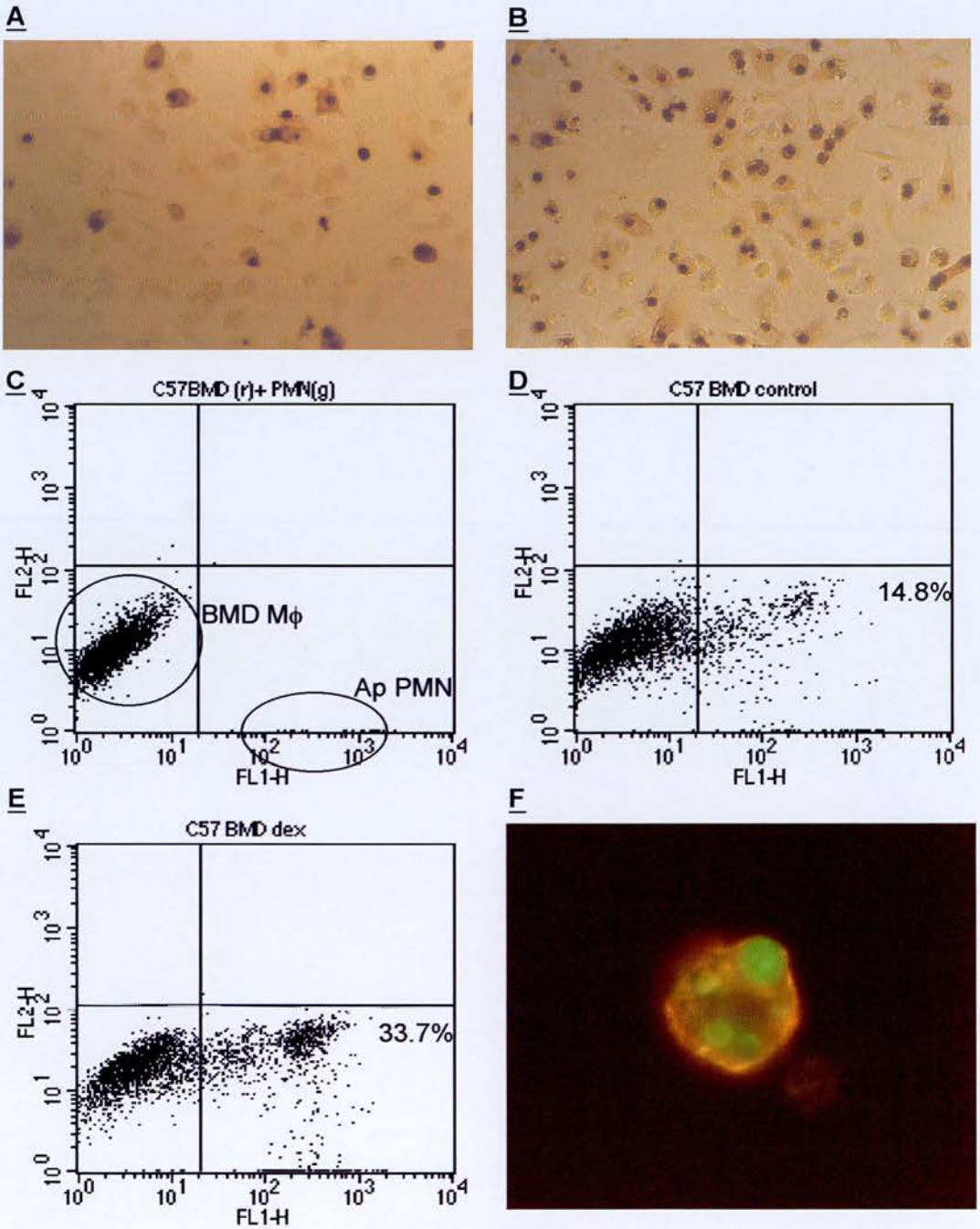


Figure 4-1: Dex treatment of Mϕs from C57BL/6 mice augments phagocytosis of aPMNs (1). A, 24h Dex treatment (200nM) prior to a 30 min *in vitro* phagocytosis assay significantly augmented the phagocytosis of aPMNs by BMD Mϕs (d7) and TEP Mϕs (harvested on d3 of TE peritonitis).
B, *in vivo*, an i.p injection of Dex (200nM) on d2 of TE peritonitis augmented the ability of d3 TEP Mϕs to ingest i.p administered aPMNs during a 10 min phagocytosis assay. Values are Mean +/- SEM of counts of 600 Mϕs from at least 4 separate experiments carried out in duplicate. *P<0.05, **P<0.001 by ANOVA.


A
B
C
C57BMD (r)+ PMN(g)
BMD Mϕ
Ap PMN
FL2-H
FL1-H
D
C57 BMD control
14.8%
E
C57 BMD dex
33.7%
F

(previous page)

Figure 4-2: Dex treatment of Mϕs from C57BL/6 mice augments phagocytosis of aPMNs (2). Untreated d7 BMD Mϕs (A), and Mϕs treated with Dex (200nM) for 24h (B), were assayed for phagocytosis *in vitro*. Dex increased both the percentage of Mϕs that phagocytosed, and the number of aPMNs that individual Mϕs ingested (phagocytic index). Ingested PMNs were selectively stained for myeloperoxidase activity.
C, F4/80 labelled BMD Mϕs and CM-Green labelled aged PMNs separated on the FL-2 and FL-1 axis respectively by flow cytometry analysis. Phagocytosis levels were determined by the percentage of gated cells that shifted into the red/ green positive quadrant of the dot-plot. E, Dex-treated Mϕs ingested more aPMNs than control cells (D). F, Photograph shows a TEP Mϕ recovered after a 10 min *in vivo* phagocytosis assay and counterstained for F4/80 expression to aid visualisation under fluorescence.

For the *in vivo* phagocytosis assay, 200nM Dex treatment was administered by i.p injection 2d day after the onset of TE peritonitis. Exogenous aPMNs stained with CM-Green dye were injected i.p into the Dex treated mouse on d3 (refer to in section 2.2-10.2). Typically, aged PMNs were introduced at a ratio of 3:1 (aged PMNs:Mϕ) and after 10 min cells were harvested. The percentage of TEP Mϕs that ingested aPMNs was approximately 2.5-fold greater in cells recovered from Dex-treated mice than control mice (Fig.4-1B). Figure 4-2F shows an F4/80 stained, Dex-treated TEP Mϕ containing a number of aPMNs after a 10 min *in vivo* assay.

A flow cytometry based protocol was also used to quantify phagocytosis of aPMNs within Mϕ populations. BMD Mϕs and aged PMNs separate on a 2-colour plot when labelled with F4/80 and CM-Green respectively (Fig.4-2C). The comparative shift of a population of cells into the red/ green quadrant is representative of phagocytosis. 24h Dex treatment confers a phagocytic effect upon a subpopulation of the Mϕs (33.7%) gated in the F4/80 population (Fig.4-2E), approximately double that observed in control cells (Fig.4-2D).

4.2-2 Macrophage 11β-HSD1 activity is inhibited by carbenoxolone

For many of the experiments in this chapter, the effect of 11β-reductase activity, and the consequence of its inhibition, on Mϕ phagocytic function was demonstrated. Derivatives of liquorice (such as glycyrrhetinic acid) and carbenoxolone (the hemisuccinate of glycyrrhetinic acid) have previously been shown to inhibit 11β-HSD activity in human and murine cells (Monder, Stewart et al. 1989; Stewart, Wallace et al. 1990).

Monolayers of d3 TEP Mϕs were pre-treated with increasing concentrations of carbenoxolone for 1h before commencement of 11β-reductase assays. No significant 11β-reductase inhibition was observed at concentrations of carbenoxolone below 10^{-6}M (Fig.4-3). Approximately 60% inhibition was detected with 10^{-5}M, increasing to almost complete inhibition with a concentration of 10^{-4}M (Fig.4-3).

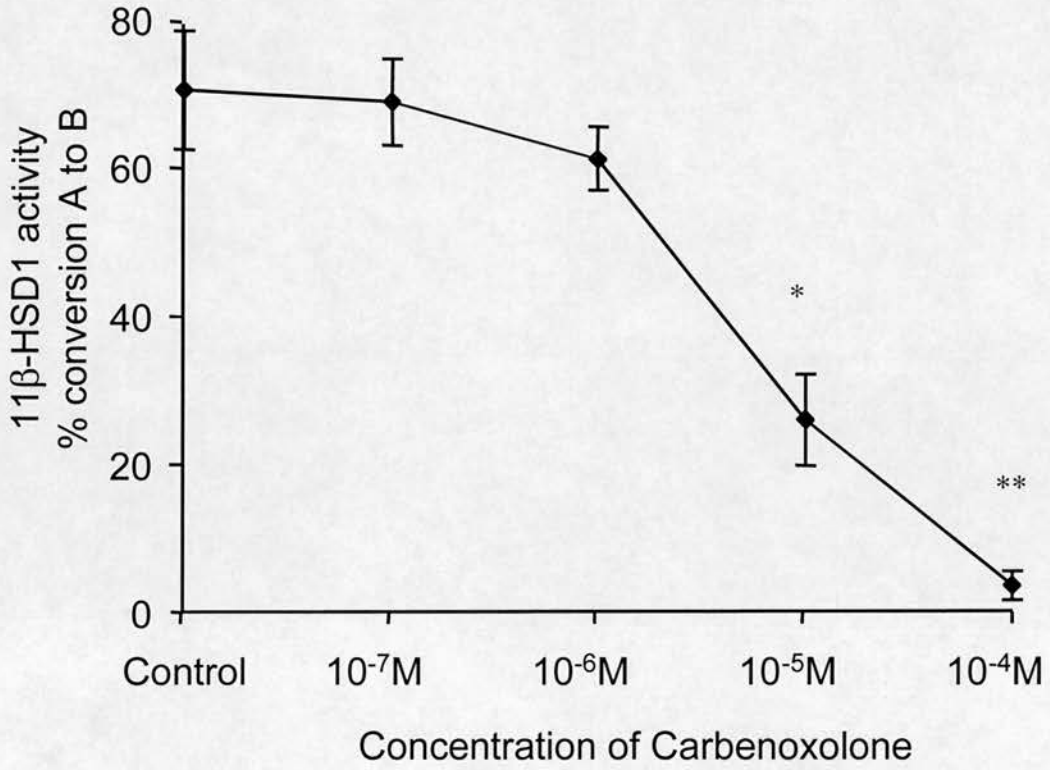


Figure 4-3: Carbenoxolone, inhibits 11β-reductase activity in TEP Mϕs. 10^6 TEP Mϕs harvested at d3 were pre-treated for 1h with carbenoxolone (Cx) at various concentrations (as indicated) then 11β-reductase activity measured over 24h. 11β-HSD1 activity is reported as % conversion of 200nM A to B. At 10^{-6}M or below, Cx did not significantly inhibit reductase activity, whereas approximately 60% and 100% inhibition was seen with concentrations of 10^{-5}M and 10^{-4}M, respectively. Values shown are mean +/- SEM of 3 different mice, measured in duplicate. *P<0.05, **P<0.001 (ANOVA), compared to control.

4.2-3 Physiological glucocorticoids augment the phagocytosis of apoptotic neutrophils by macrophages

Dose response studies performed by Liu showed that phagocytosis was maximally augmented by Dex over 24h at a concentration of 200nM (Liu, Cousin et al. 1999). 24h incubation with either B or A at 200nM was as effective in augmenting phagocytosis of aPMNs by TEP Mϕs as 200nM Dex over the same time period (Fig.4-4). The assays were repeated using high physiological levels of physiological steroids comparable to those detected in plasma during stress (Harris, Kotelevtsev et al. 2001). Either 20nM B or A increased phagocytosis, although the effect of A was suggestive since significance was not established (Fig.4-4). Lui et al showed that greater augmentation of phagocytosis was possible with the same concentration of Dex if the duration of exposure to GC increased (Liu, Cousin et al. 1999). In the case of A, significant increases were observed at concentrations of 20nM and less in later experiments when the duration of exposure was increased to 48h (data not shown, refer to 6.2-1).

4.2-4 Carbenoxolone inhibits 11-dehydrocorticosterone, but not corticosterone augmentation of phagocytosis by macrophages

To investigate the specificity of 11β-HSD1 action on the effect augmented by A, 11β-reductase activity was inhibited with carbenoxolone. Pre-treatment of TEP Mϕs for 1h with 10^{-4}M carbenoxolone before GC addition, abolished the effect of A upon phagocytosis of aPMNs, but had no effect on B augmentation of phagocytosis (Fig.4-4). Inhibition of 11β-reductase activity by carbenoxolone had no effect on control of phagocytosis, although a trend towards marginally decreased phagocytosis was frequently observed with carbenoxolone treatment.

4.2-5 Physiological glucocorticoids augment phagocytosis of apoptotic neutrophils by mesangial cells

24h treatment of rat MC with either 200nM B or A augmented phagocytosis by approximately 2-fold. This increase was comparable to that resulting from Dex

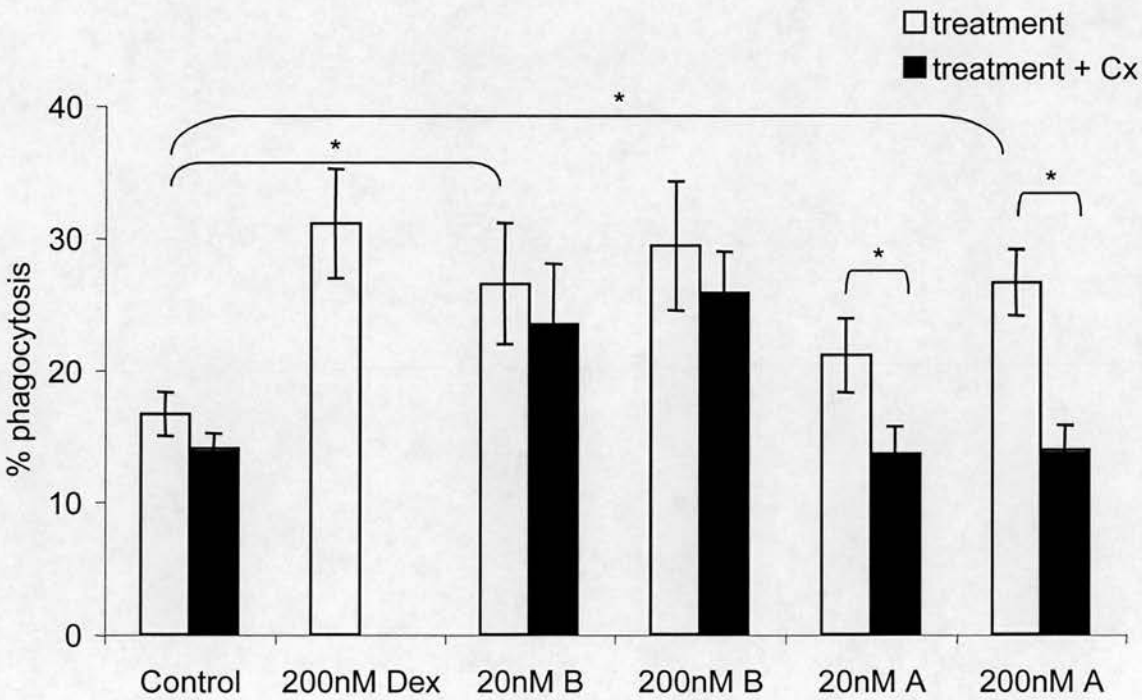


Figure 4-4: Physiological GCs augment phagocytosis. Carbenoxolone inhibits the capacity of A, but not B, to augment phagocytosis by TEP Mϕs. TEP Mϕs were harvested on d3 of peritonitis and cultured 24h in the presence of Dex, A or B. Aged PMNs were allowed to interact with the Mϕs (at a ratio of 4:1) for 30 mins, and % of Mϕs that ingested 1 or more aPMNs were assessed by microscopy. Pre-treatment for 1h with 10^{-4}M carbenoxolone (Cx) before A addition abolished the effect of A on phagocytosis, but had no effect on phagocytosis by B-treated or control cells. Values shown are mean +/- SEM of counts of 600 Mϕs from 6 mice, carried out in duplicate. *P<0.05 (ANOVA), as indicated

treatment (Fig.4-5). Note that the low levels of ingestion achieved after 2h interaction with aged PMNs is consistent with published observations (Savill, Smith et al. 1992). Inhibition of 11β-reductase activity by inclusion of carbenoxolone 1h prior to addition of A abolished the effect of A (Fig.4-5).

4.2-6 Human monocytes are responsive to corticosterone, but not 11-dehydrocorticosterone

So far this chapter has focused on the effect of GC upon mature Mϕs. In the human system, Dex treatment of monocytes has recently been shown to affect monocyte to MD Mϕ differentiation (Giles, Ross et al. 2001). The phagocytic potential of mature MD Mϕs can be increased by an initial 24h exposure of the fresh monocytes to Dex at day 0 (d0) (Giles, Ross et al. 2001). This effect was conferred before the induction of 11β-HSD1 observed on d2 of differentiation (Chapter 3). In the present study it is predicted that monocytes will have differing responses to physiological GCs, by virtue of a lack of 11β-HSD1 expression. Furthermore, it would be of interest to determine whether there may be a role for 11β-HSD1 in differentiation.

The phagocytosis of aPMNs by MD Mϕ was augmented in response to 24h GC treatment (Fig.4-6). 24h incubation with either, 1μM Dex, B or A added at d3 of differentiation increased phagocytosis by at least 2-fold. Similarly, consistent with previous studies (Giles, Ross et al. 2001), inclusion of Dex or B at d0 with fresh monocytes conferred an increased capacity for phagocytosis upon MD Mϕs at d4, even when the GCs were removed upon the standard d2 replacement of medium (Fig.4-6). However this was not the case when monocytes were treated with A. MD Mϕs were responsive to the effects of A, but monocytes were not (Fig.4-6). Monocytes do not express 11β-HSD1 so therefore do not have the ability to generate B from exogenously added A, thus phagocytosis levels remain similar to control levels.

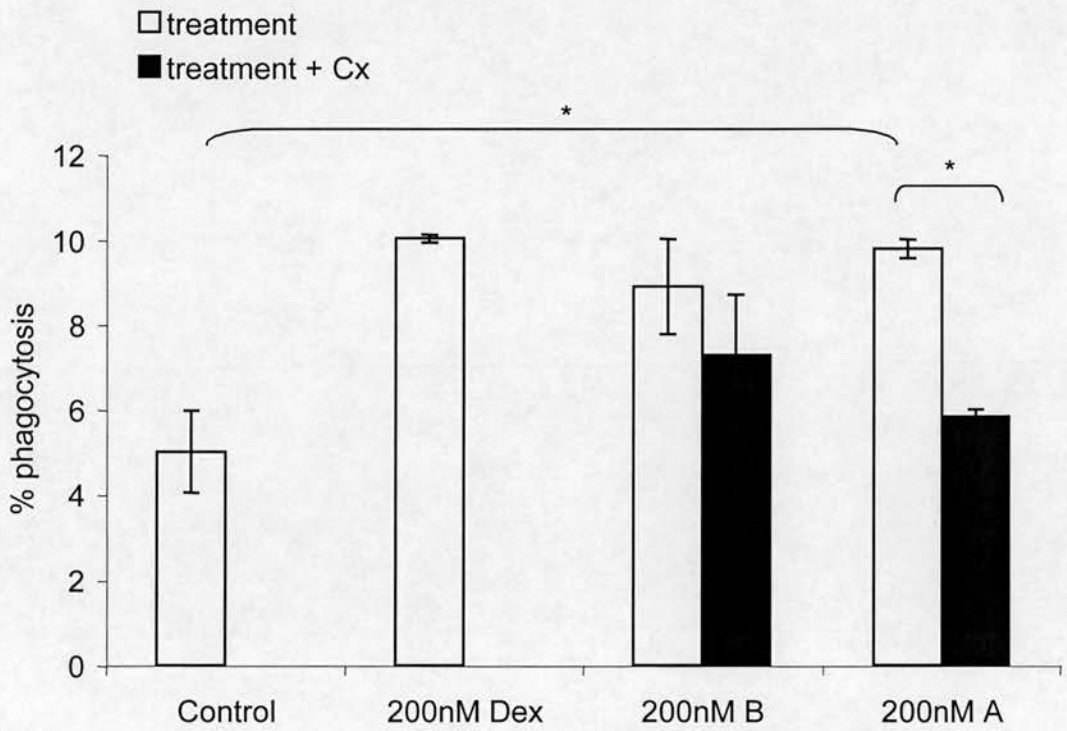


Figure 4-5: Physiological GCs augment phagocytosis. Carbenoxolone inhibits the capacity of A, but not B, to augment phagocytosis by rat MC. Rat MC were cultured for 24h in the presence of Dex, A or B. Aged PMNs were allowed to interact with the MC (at a ratio of 4:1) for 2h in the presence of 10% serum, and % of MC that had ingested 1 or more aPMNs were assessed by microscopy. Pre-treatment of MC for 1h with 10^{-4}M carbenoxolone (Cx) before GC addition abolished the effect of A upon phagocytosis of aPMNs. Values shown are mean +/- SEM of counts of at least 500 MC from 3 separate experiments, carried out in duplicate. *$P<0.05$ (ANOVA), as indicated.

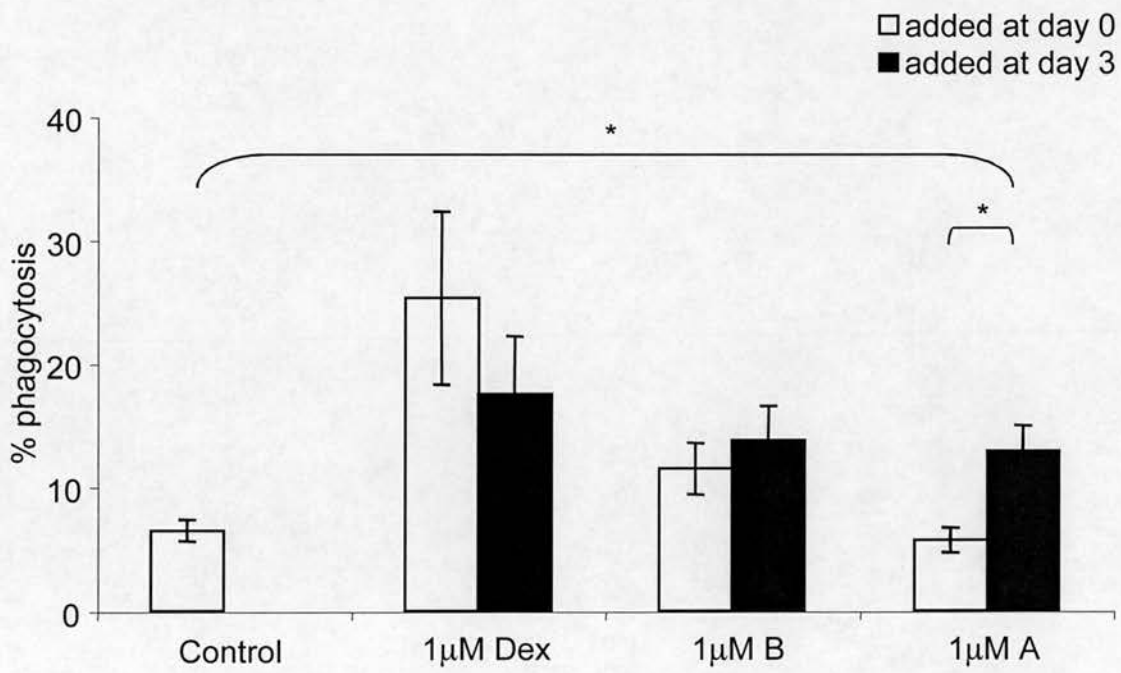


Figure 4-6: Augmentation of phagocytosis by inert A only occurs once 11β-HSD1 expression has been induced during human monocyte differentiation. GCs were added to $4X10^6$ human monocytes on either d0 or d3 of culture and phagocytosis assays performed on d4. Control cells remained untreated. Aged PMNs were allowed to interact with the MD Mϕs (at a ratio of 4:1) for 30 mins, and % of Mϕs that had ingested 1 or more aPMNs were assessed by microscopy. A treatment conferred augmentation on Mϕs when added at d3 but not d0, whereas B and Dex augmented phagocytosis when added at either time-point. Values shown are Mean +/- SEM of counts of at least 600 Mϕs of 5 different experiments, carried out in duplicate. *$P<0.05$ (ANOVA), as indicated.

4.2-7 Glucocorticoid treatment of monocytes promotes acquisition of phagocytic competency

It was of interest to determine if the availability of GC early during monocyte differentiation affected the rate at which MD Mϕs become competent to phagocytose aPMNs. Human MD Mϕs did not acquire the ability to phagocytose aPMNs until d3 of differentiation (Fig.4-7). In contrast, monocytes treated with 1μM Dex or B attained this ability by d2 of differentiation. Interestingly, monocytes treated with A did not differ from controls in acquisition of phagocytic competency (Fig.4-7).

4.3 DISCUSSION

Previous work has shown that synthetic GCs augment the phagocytic capacity of Mϕs and MC for aPMNs (Liu, Cousin et al. 1999). In the present study, these findings have been extended to show that the physiological GC, B can confer a similar effect. Moreover, this effect can also be conferred by the reactivation of B from inert A by the action of phagocyte 11β-reductase, thus suggesting that, *in vitro* at least, Mϕ and MC clearance of aPMNs can be facilitated by virtue of 11β-HSD1 activity. *In vivo*, however such an effect may only be conferred in instances where A is present and free B is low.

Work in this chapter validates Liu's findings (which were in BALB/C mice) in the C57BL/6 strain of mouse. Despite apparent differences in abilities to elicit inflammatory responses (Mills, Kincaid et al. 2000), no differences were observed between Mϕs from BALB/C or C57BL/6 mice in their pro-phagocytic response to Dex. Furthermore, this response was validated *in vivo*, using a model of sterile thioglycollate-induced peritonitis. 24h i.p Dex treatment led to a 2-fold augmentation of phagocytosis of aPMNs. Not only did GC treatment increase the number of Mϕs capable of ingestion but it also augmented the capacity of individual Mϕs to ingest multiple aPMNs. Indeed, in *in vivo* phagocytosis assays, Mϕs recovered after only 10 min exposure to aPMNs often contained multiple phagocytic bodies and, when longer assays were performed, rapid degradation of the ingested aPMN was observed (data not shown). Therefore it is

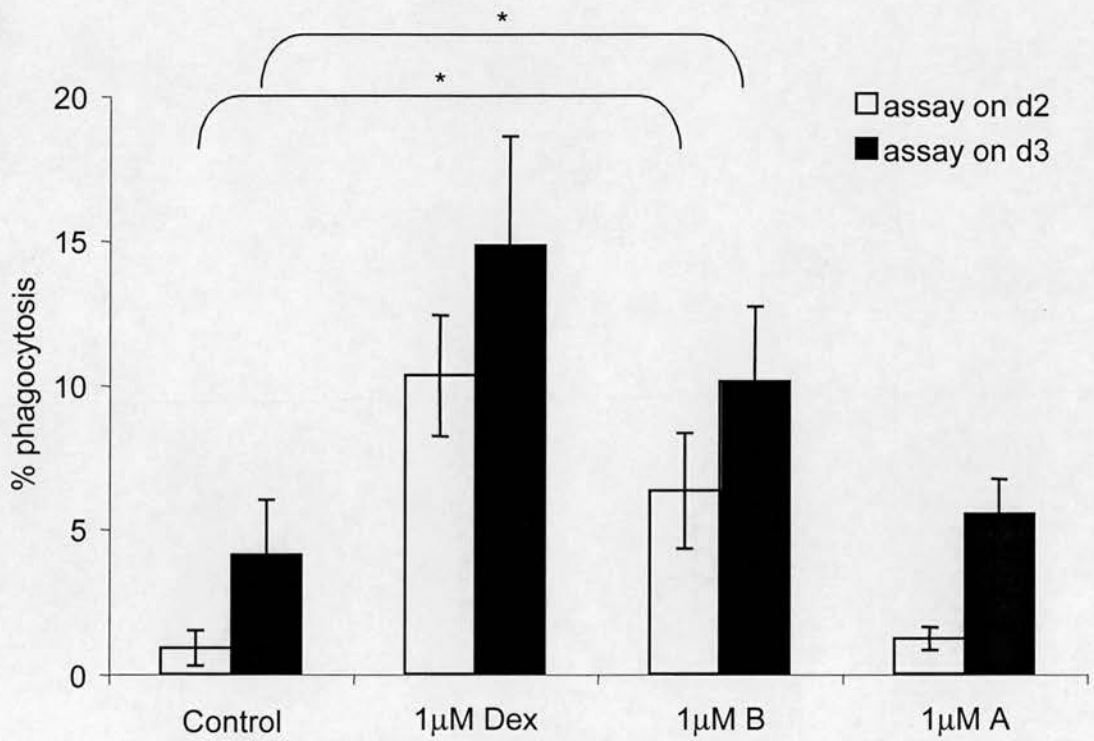


Figure 4-7: GC treatment of human monocytes promotes the attainment of phagocytic competency for aPMNs. Freshly isolated human monocytes were cultured in the presence of 1μM Dex, B or A. Aged PMNs were allowed to interact with the Mϕs (at a ratio of 4:1) for 30 mins at d2 and d3 of monocyte to Mϕ differentiation, and % of Mϕs that had ingested 1 or more aPMNs were assessed by microscopy. Untreated Mϕs (Control) attained the ability to ingest aPMNs on d3 whereas Mϕs treated with Dex or B from d0 attained this ability by d2. Treatment with A did not advance this rate of phagocytic competency, and phagocytosis levels did not differ from control. Values are Mean +/- SEM of counts of at least 600 Mϕs of 3 separate experiments carried out in duplicate. *P<0.05 (ANOVA), compared with control on the same day.

reasonable to speculate that increasing phagocytic capacity, *in vivo*, would represent a possible therapeutic approach towards promoting resolution of inflammation.

Quantifying phagocytosis levels by flow cytometry proved to be an inconsistent method of analysis. This was largely due to the levels of auto-fluorescence emitted by the Mϕs, in particular TEP Mϕs. Nevertheless, this method has been validated by others (Brown, Heinisch et al. 2002) and may be better suited for assay of human MD Mϕ phagocytosis rather than mouse. Unfortunately therefore, this form of phagocytosis analysis had to be abandoned in favour of the time-consuming but accurate method of counting by eye.

A potentiating effect similar to that of Dex was observed after 24h or 48h treatment of phagocytes, *in vitro* with either B or A, respectively. The action of A requires 11β-HSD1, as it was abolished by pre-treatment with the 11β-HSD inhibitor, carbenoxolone. Importantly, this effect was apparent with 48h exposure to concentrations within the physiological range such as 20nM. The equipotency of B to Dex was somewhat surprising given that B is more readily metabolised by cells than Dex. However, it is possible that Dex is subject to active transport from within Mϕs by drug-transporting glycoproteins such as MDR3, effectively lowering the intracellular Dex concentration (Schinkel, Smit et al. 1994). However, despite the potency of physiological GCs in this culture system, *in vivo* their effect would be compromised by diffusion, CBG binding and metabolism by 11β-HSD2 unless the pro-phagocytic effect was specifically targeted to Mϕ populations.

Longer incubations with carbenoxolone (≥48h) appeared detrimental to the cells, particularly when added to differentiating monocytes (unpublished observation). In view of the fact that 11β-HSD1-deficient Mϕs were viable (refer to section 3.2-3) cell loss during this prolonged treatment is most likely due to other pathways inhibited by carbenoxolone rather than a consequence of 11β-HSD inhibition (Stewart, Wallace et al. 1990).

Recently published work from our group suggests that, in addition to augmenting phagocytosis of differentiated MD Mϕs, Dex can confer an effect on the differentiation

process itself, even if present during only the first 24h only (Giles, Ross et al. 2001). In the present study it was shown that MD Mϕs could be programmed to be pro-phagocytic by exposure to Dex or B whilst still at the monocytic stage (despite medium change and removal of GCs at d2), whereas exposure to A was without effect. *In vitro*, A only conferred enhanced phagocytosis on MD Mϕs when added after appearance of 11β-HSD1 induction on d2. Additionally, exposure to GC also promoted the attainment of phagocytic competency for aPMNs. For example monocytes treated with Dex or B were able to ingest aPMNs 24h earlier than control or A treated cells. This raises the question of whether early induction or over-expression of monocyte 11β-HSD1 by differentiation agents or by transgene is possible? Such a strategy would target the pro-phagocytic effects of B earlier to differentiating monocytes yet may only necessitate non-toxic physiological concentrations of A – either by administration of A therapeutically, or by utilising the endogenous supply.

Liu et al showed that Dex specifically promoted phagocytosis of apoptotic cells (including PMNs, eosinophils and Jurkat T lymphocytes) by various populations of phagocytes that usually employ different phagocytic receptors (such as $\alpha_v\beta_3$) to engage apoptotic cells (Liu, Cousin et al. 1999). Despite the "pan-phagocyte" effect of Dex, the increase in phagocytosis was specific for apoptotic cells since the ingestion of other classes of 'feed' such as opsonized erythrocytes was unaffected by Dex (Liu, Cousin et al. 1999). Moreover, as seen with untreated phagocytes, GC-treated phagocytes also retain the ability to suppress the release of pro-inflammatory mediators after phagocytosis of apoptotic cells (Meagher, Savill et al. 1992; Liu, Cousin et al. 1999). In comparison, the ingestion of opsonized zymosan elicits a pro-inflammatory response from GC-treated phagocytes (Liu, Cousin et al. 1999), and thus the suppression of Mϕ secretion seen after the ingestion of apoptotic cells was not due to a generalised GC-suppression of phagocyte responses. It is hypothesised that these novel anti-inflammatory properties of pharmacological GCs combine to promote the successful resolution of an inflammatory response (Liu, Cousin et al. 1999). Could endogenous B play an equivalent role during an acute, self-resolving infection? In this present study,

data are presented showing that phagocytes possess the machinery to generate physiological GC from its inert precursor, and that, given sufficient substrate, this amplification can facilitate an increased clearance of aPMNs *in vitro*. This action is not unique to any single class or species of phagocyte and appears to be induced upon differentiation. It will be of great interest to determine whether this phenomenon takes place *in vivo*, and to determine the extent to which 11β-HSD1 activity is controlled locally by the inflammatory environment.

4.4 SUMMARY

- Mϕs from C57BL/6 mice augmented phagocytic capacity for aPMNs in response to Dex treatment.
- Physiological GCs were effective in augmenting phagocytosis of aPMNs by murine Mϕs, human monocyte-derived Mϕs and rat mesangial cells.
- Carbenoxolone inhibited 11β-reductase activity in Mϕs and abrogated the increased phagocytic levels seen in response to A treatment.
- Human monocyte capacity and acquisition of capacity to ingest aPMNs was both accelerated and increased by active GC.
- Augmentation of phagocytosis by inert A only occurred once 11β-HSD1 expression had been induced during human monocyte differentiation.

Chapter 5:

Modulation of 11β-HSD1 activity

5.1 INTRODUCTION

The data presented in Chapter 3 suggested that 11β-HSD1 activity in TEP Mϕs may have been greater than in RP Mϕs. Since variation in cell number and viability were unlikely to account for the differences, it appeared possible that 11β-HSD1 expression was influenced by the inflammatory environment. Reports of 11β-HSD1 modulation in the literature show that hepatic, brain and adipose 11β-HSD1 mRNA levels can be influenced by a number of factors including TNFα (refer to section 1.4-3). The aim in this chapter was to determine whether Mϕ 11β-HSD1 expression could be influenced by inflammatory mediators – in particular cytokines.

During the course of this thesis, a report of cytokine regulation of Mϕ 11β-HSD1 was published (Thieringer, Le Grand et al. 2001). 11β-HSD1 expression in MD Mϕs was shown to be increased by the Th2 cytokines, IL-4 and IL-13, an effect that was abrogated in the presence of pro-inflammatory IFN-γ (Thieringer, Le Grand et al. 2001). In addition it was also reported that LPS up-regulated 11β-HSD1 expression in the Mϕ-like cell line, THP-1 (Thieringer, Le Grand et al. 2001). In contrast, in rat MC 11β-HSD1 mRNA was increased by pro-inflammatory cytokines TNF-α and IL-1β (Escher, Galli et al. 1997).

Thus, both pro- and anti- inflammatory cytokines have been shown to regulate 11β-HSD1 mRNA, albeit in different phagocyte cell types. Given the suggestion of increased 11β-HSD1 activity in inflammatory Mϕs compared to resident Mϕs it was important to examine the regulation Mϕ 11β-HSD expression. 2 systems were investigated: (i) 11β-HSD1 activity in Mϕs elicited into the peritoneum during the course of peritonitis and, (ii) function consequences of cytokines on differentiation and phagocytic ability during differentiation of human monocytes to MD Mϕs.

5.2 RESULTS

5.2-1 11β-HSD1 is up-regulated in inflammatory peritoneal cells during sterile peritonitis

To determine whether 11β-HSD1 expression changed during TE peritonitis, 11β-reductase was assayed in cultured peritoneal cells harvested at various times after i.p thioglycollate injection.

In an initial experiment 11β-HSD1 activity increased rapidly after thioglycollate injection, so that at d1, cells fully converted 200nM A to B over a subsequent 24h, whereas RP Mϕs only converted 30% of the added A (Fig.5-1). Conversion levels remained close to 100% until d3, after which 11β-HSD1 activity decreased to a level comparable to that of RP Mϕs (Fig.5-1). 11β-HSD1 levels in this assay were clearly saturated by d1, therefore to further investigate the rapid induction of 11β-HSD1 activity and determine the magnitude of induction, cells taken 4h after thioglycollate injection were adhered to plastic, and changes in 11β-HSD1 activity assayed following 4h, 8h and 24h incubation. Compared to RP Mϕs, the TE peritoneal cells showed a rapid and dramatic increase in 11β-HSD1 activity after 4h culture (≥12-fold) (Fig.5-2). With longer assays, the 200nM A was completely converted to B, in contrast to RP Mϕs which converted just 30% in a 24h assay (Fig.5-2).

5.2-2 Identification of cell type responsible for 11β-HSD1 induction

The rapid and marked induction of 11β-reductase activity during d1 of peritonitis was surprising. It was therefore of interest to discover whether this increase in activity occurred in cell populations resident to the peritoneal cavity or whether it was due to cells recruited during peritonitis. Accordingly, it was investigated whether the RP Mϕ population or the recruited cell populations (predominantly monocytes and PMNs) (reviewed Chapter 1, section 1.4) were responsible for the increased 11β-reductase activity.

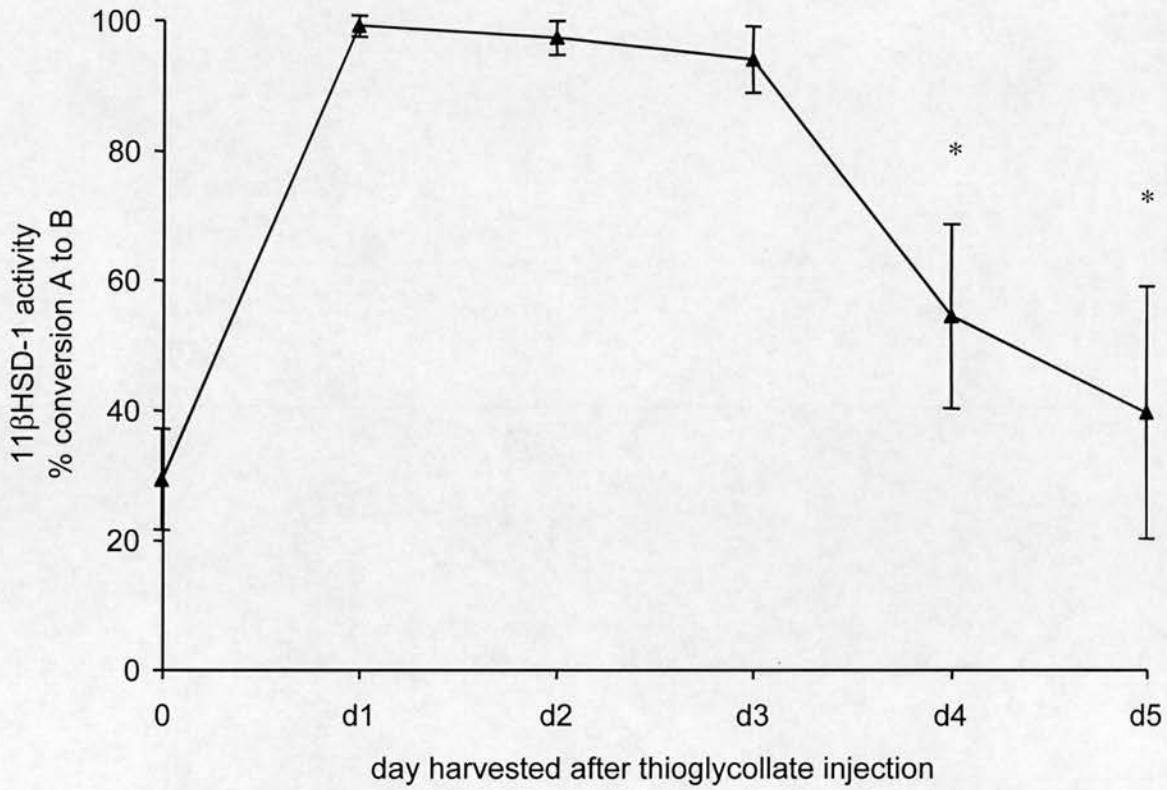


Figure 5-1: 11β-reductase activity in murine peritoneal cells during TE peritonitis. Peritoneal cells were lavaged from mice at d1-5 after 3% thioglycollate injection. 11β-HSD1 activity is reported as % conversion of 200nM A to B by 10^6 cells over a 24h period. Cells harvested at d0 converted 30% of available substrate over 24h. 1d after thioglycollate injection conversion levels increased to 100% and remained maximal until d3. By d5 the conversion level had returned to a level comparable with that of d0 (unstimulated peritoneum). Values are Mean +/- SEM of conversion levels of cells derived from 4 mice at each time-point. *$P<0.05$ (ANOVA), compared to d3.

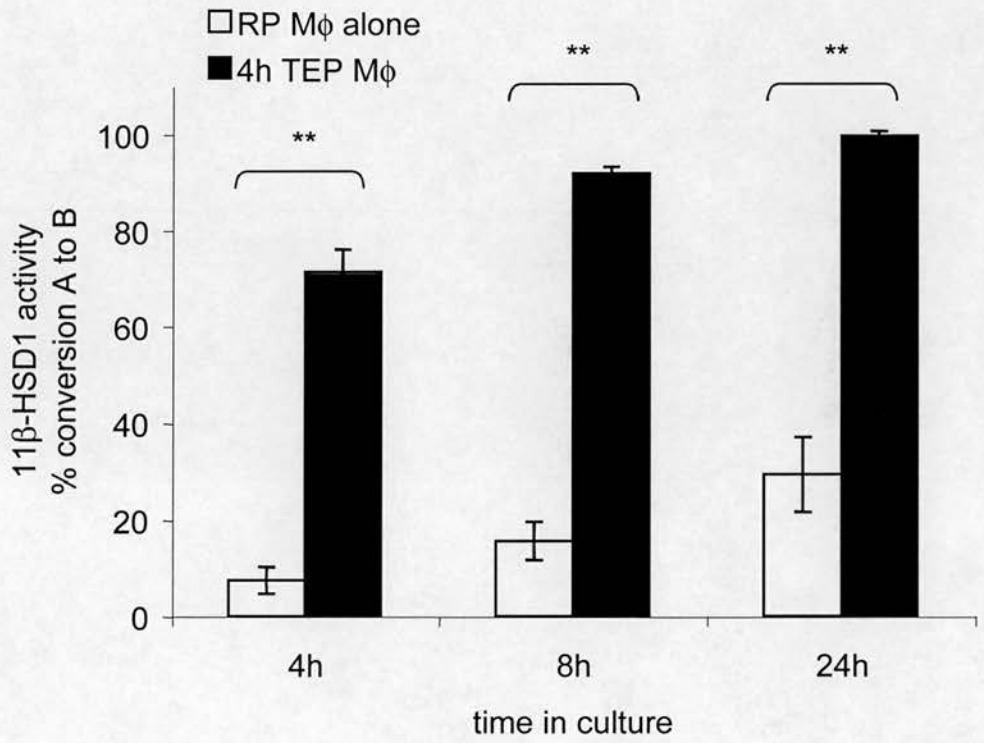


Figure 5-2: 11β-reductase activity is rapidly induced during the early stages of TE peritonitis. Conversion rates were compared between RP Mϕs and peritoneal cells harvested 4h after i.p injection of thioglycollate. 11β-HSD1 activity is reported as % conversion of 200nM A to B by 10^6 adherent cells at specified times. Values shown are Mean +/- SEM of cells derived from 4 mice carried out in duplicate. **P<0.001 (ANOVA), as indicated.

5.2-2.1 Effect of pro-inflammatory supernatant on 11β-HSD1 activity:

It was of interest to see whether the addition of thioglycollate alone, or factors present at the inflamed site was necessary for the increased 11β-reductase activity observed during peritonitis. Both RP Mϕs and murine WBC demonstrate 11β-reductase activity (refer to section 3.2-3) and therefore these cells were chosen to represent the resident or recruited populations (that may contribute to the increased activity), respectively.

Cell-free inflammatory supernatant was generated by the centrifugation of undiluted peritoneal fluid collected 4h after i.p thioglycollate injection (refer to section 2.2-8.2). Addition of supernatant to cultures of RP Mϕs and WBC caused the rapid induction of 11β-HSD activity (Fig.5.3B and C). Furthermore, 11β-HSD activity was unaffected by preparations of thioglycollate (Fig.5.3 B and C), and therefore 11β-HSD1 induction was likely to be mediated by an endogenous substance rather than as a direct effect of inflammatory agent. In summary, both RP Mϕs and WBC have an inducible 11β-HSD1 that can be up-regulated by pro-inflammatory mediators. Also, this data suggest that 11β-reductase activity can be re-induced in mature 3d inflammatory TEP Mϕs by addition of this supernatant (Fig. 5-3A).

Time only allowed an initial experiment to begin to identify the 11β-HSD1-inducing mediator(s) within the peritoneal fluid. By ELISA, it was possible to detect low levels of TNFα in diluted peritoneal fluid (collected from the peritoneal cell lavages) (Fig.5-4). TNFα was detected between 4h and 1d after the onset of peritonitis, but was undetectable between d2 and d4 (Fig. 5-3). The significance of this finding has yet to be explored, as is monocyte/ TEP Mϕ 11β-reductase activity after the direct addition of recombinant IL-4.

5.2-2.2 Cell sorting of monocytes and PMNs:

Since 11β-reductase activity was inducible in WBC, the hypothesis that murine monocytes up-regulate 11β-HSD1 expression in response to differentiation (in an analogy to human monocytes) was explored. Thus, it was necessary to distinguish the monocytes from the PMNs and therefore, attempts were made to sort peritoneal cells

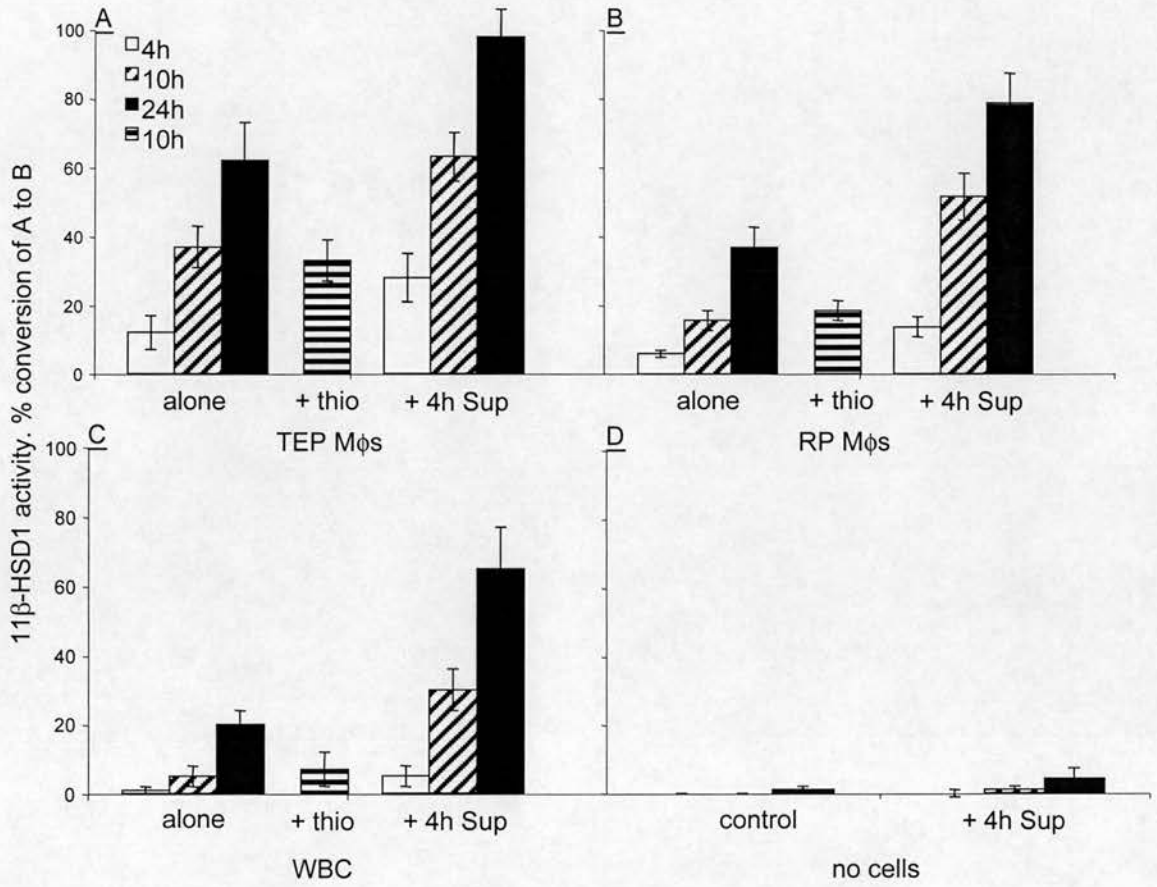


Figure 5-3: Pro-inflammatory supernatant induces 11β-HSD1 activity in Mϕs and WBC. Supernatant (4h Sup) was generated by the centrifugation of peritoneal exudate collected 4h after onset of TE peritonitis. 50µl was added to 10^6 cells and 11β-HSD1 activity measured at 4h, 10h and 24h after addition. Similarly, 50µl 3% thioglycollate (thio) was added, and activity measured at 10h. 11β-HSD1 activity is reported as % conversion of 200nM A (added at t=0) to B. A, B and C, thio had no effect on 11β-HSD1 activity at 10h, whereas 4h Sup appeared to induces 11β-reductase activity at 4h, 10h and 24h.

D, no conversion was detected in the absence of cells (control), and the supernatant had no intrinsic 11β-reductase activity. Values shown are Mean +/- range from 2 experiments done in duplicate. Significance not established.

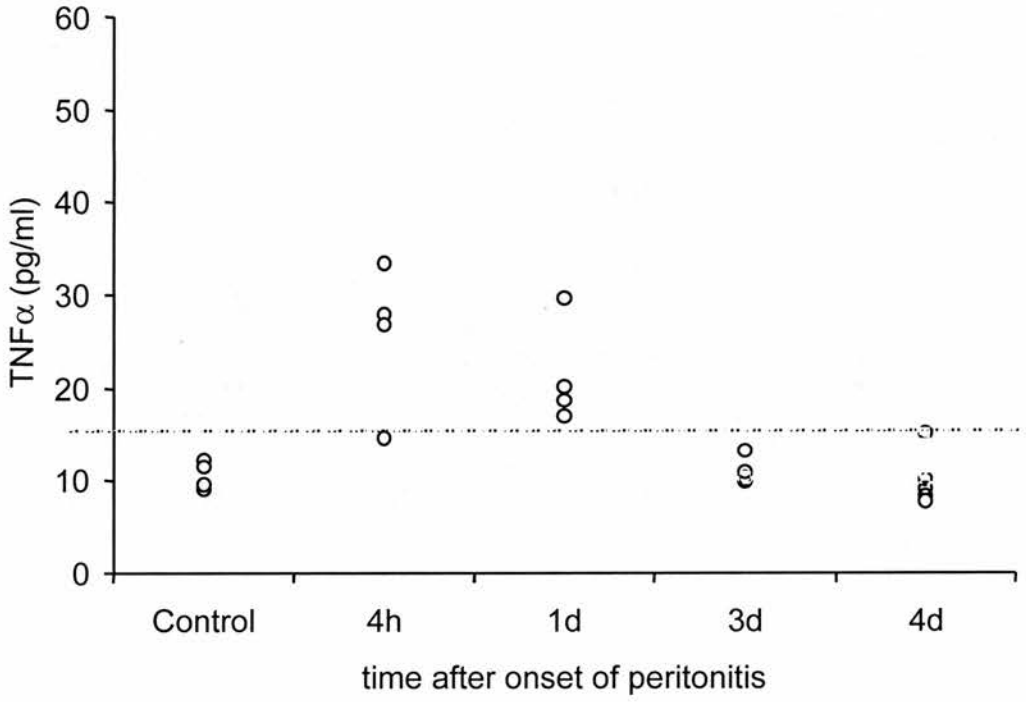


Figure 5-4: TNFα is detected in peritoneal lavages taken 4h and 1d after the onset of TE peritonitis. 3ml peritoneal supernatant was returned from the 4ml PBS bolus used to harvest peritoneal cells. Cells and debris were removed by centrifugation, and TNFα concentrations were measured by ELISA. TNFα was present at detectable levels in samples taken 4h and 1d after i.p thioglycollate injection. The dotted line indicates detection threshold of the ELISA kit.

collected 4h after the onset of peritonitis into monocyte/Mϕ and PMN populations by FACSVantage.

It is particularly difficult to separate monocytes from PMNs as they share many surface molecules including GR-1 (Lagasse and Weissman 1996). This experiment attempted to sort these cells on the basis that by 4h of peritonitis, the infiltrating monocytes and PMNs would be sufficiently differentiated to be distinguishable by expression of their mature surface markers, F4/80 and GR-1 respectively. Peritoneal cells harvested 4h after thioglycollate injection separate into 2 populations of cells (based on forward and side scatter properties), both of which contain cells positive for anti-F4/80 and anti-GR-1 (Fig.5-5). Interestingly, a clear difference in fluorescence was seen between GR-1 positive cells in R1 and R2, whereas no difference was observed in F4/80 positive cells between the 2 populations (Fig.5.5B and C).

To determine which, if either of the cell populations contained high levels of 11β-HSD1 expression, cells were sorted from R1 or R2 on the basis of F4/80 or GR-1 expression and were bioassayed for 11β-HSD1 activity over 24h. No differences in 11β-reductase activity were seen between F4/80 or GR-1 positive cells within or between R1 and R2 (data not shown). Furthermore, microscopic examination clearly showed that the GR-1 positive cells contained significant numbers of Mϕs, and therefore could not be representative of a PMN population.

However, many of the recovered cells were irreparably damaged by the sorting process and were either apoptotic or had fragmented- despite the use of a medium buffered with serum to minimise shear forces. After 1 repeat with similar observations, it was decided that cell sorting of peritoneal cells on the basis of F4/80 and GR-1 was not a viable method for determining the source of 11β-HSD1 activity.

5.2-2.3 Monocyte depletion during peritonitis:

Using a different approach, it was decided to establish the 11β-reductase contribution of the infiltrating monocytes indirectly by eliminating them from TE peritonitis. The diphtheria-toxin receptor (DTR) mouse was generously provided by Dr. Jeremy Hughes

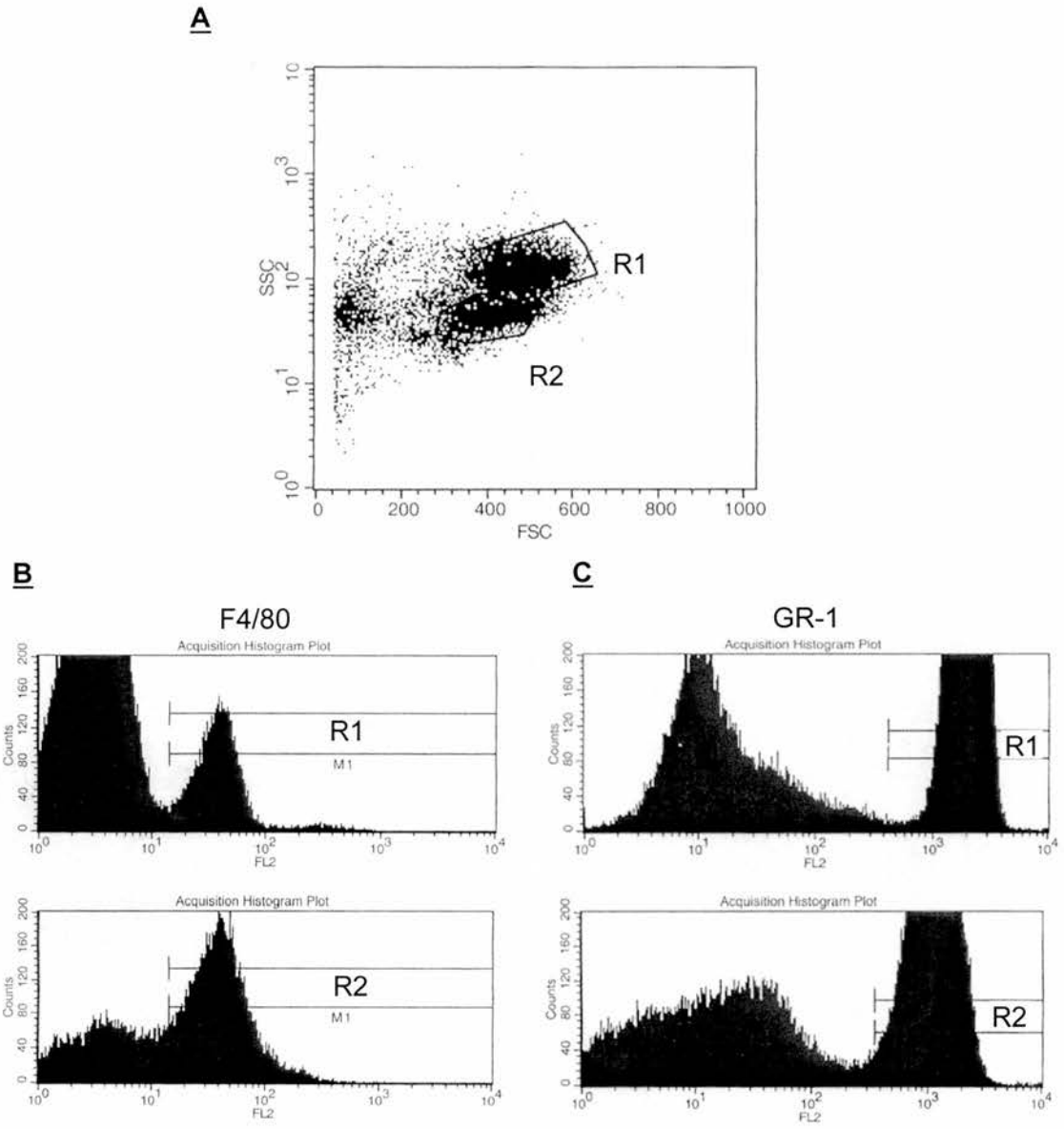


Figure 5-5: Cell sorting of F4/80 and GR-1 positive cells taken 4h after the onset of TE peritonitis. Peritoneal cells were harvested after 4h of TE peritonitis and stained for surface expression of either F4/80 or GR-1. A, 2 populations, R1 and R2 separated on the basis of size. However both R1 and R2 contained cells that were F4/80 positive (B), or GR-1 positive (C). The gate M1 represents a shift greater than that of isotype control. C, the cells in R1 had a greater level of GR-1 staining than those in R2. Data representative of 50,000 cells collected per gate in 2 separate experiments.

CIR, in which it is possible to temporarily delete the majority of circulating monocytes by intra-venous exposure to diphtheria toxin (DT) (refer to section 2.2-4.4).

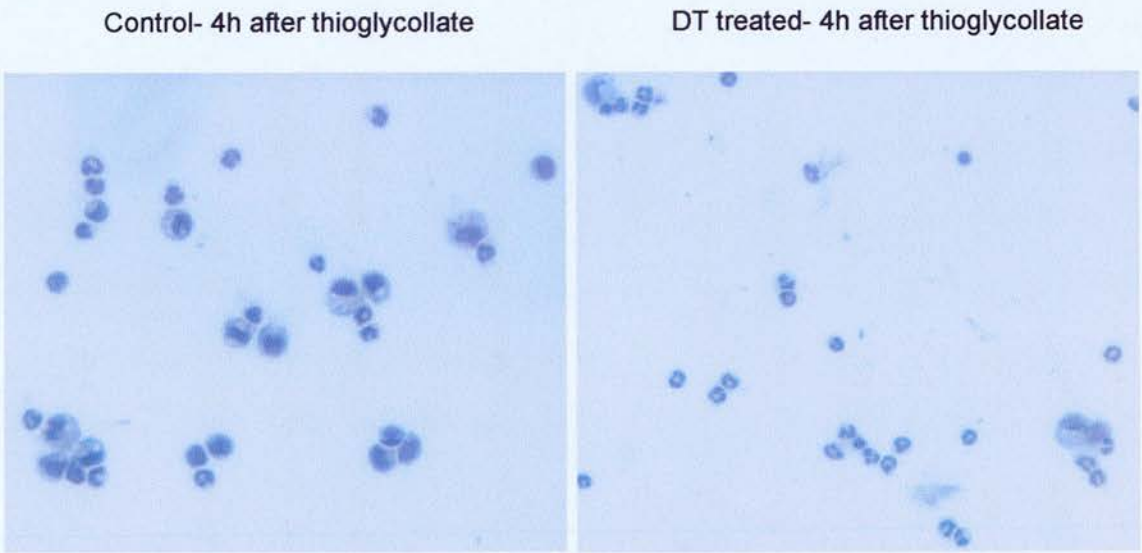
DT was administered, followed 24h later by i.p thioglycollate injection. Cells collected 4h after onset of peritonitis were assayed for 11β-reductase activity and viewed by microscopy. Figure 5-6A shows that DT pre-treatment reduced (but did not completely eliminate) the proportion of recruited monocytes by ~95%. Cells taken at this time-point were cultured and 11β-reductase activity was assayed 4h and 20h after plating. No significant differences were seen in 11β-reductase activity at either time-point (Fig.5-6B). Even though the monocyte population was not completely eliminated, these data suggest that the increased 11β-reductase activity was present in either the infiltrating PMNs or the activated RP Mϕ population.

5.2-3 Phagocytosis of apoptotic neutrophils by macrophages is apparently augmented by inflammatory supernatant

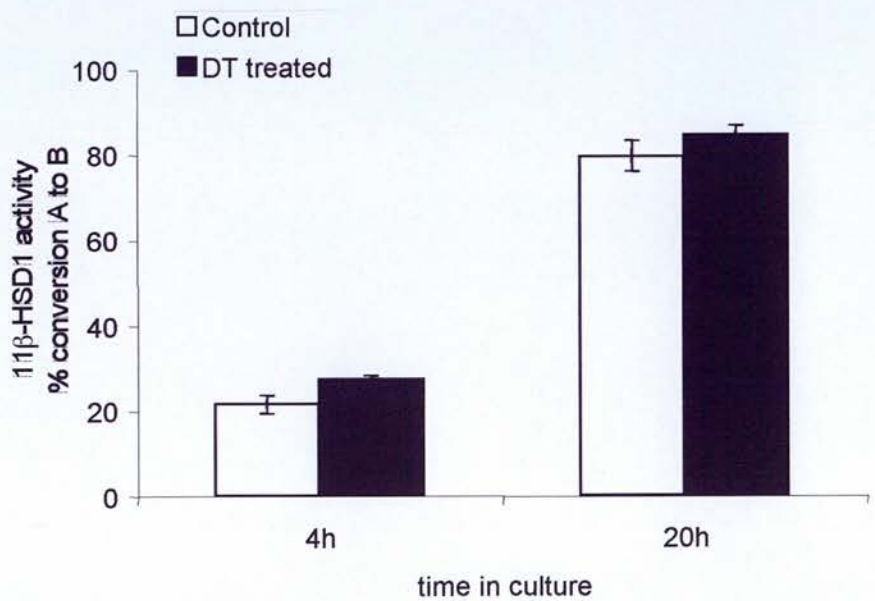
Data from the present study suggests that 11β-reductase activity within inflammatory TEP Mϕs was re-induced upon exposure to inflammatory supernatant (Fig.5-3A). Within in a few hours, 10^6 TEP Mϕs converted virtually 200nM A into B (100% conversion, see Fig. 5.3A). It was of interest to discover if this increased rate of conversion (and thus longer exposure of cells to B) could confer an even greater phagocytic potential upon TEP Mϕs, and therefore whether induction of 11β-HSD1 activity is relevant to phagocytosis.

3d TEP Mϕs were treated with A and/ or inflammatory supernatant for 24h before a 30min phagocytosis assay was carried out. Control cells had no treatment (Fig.5-7A). As expected, phagocytosis was augmented above control levels by treatment with A (Fig.5-7C). Interestingly, a similar augmentation was seen after exposure to inflammatory supernatant alone (Fig.5-7B). However, addition of both together appeared to be additive, (Fig.5-7D and E). Due to difficulty of maintaining sterility during collection of

A

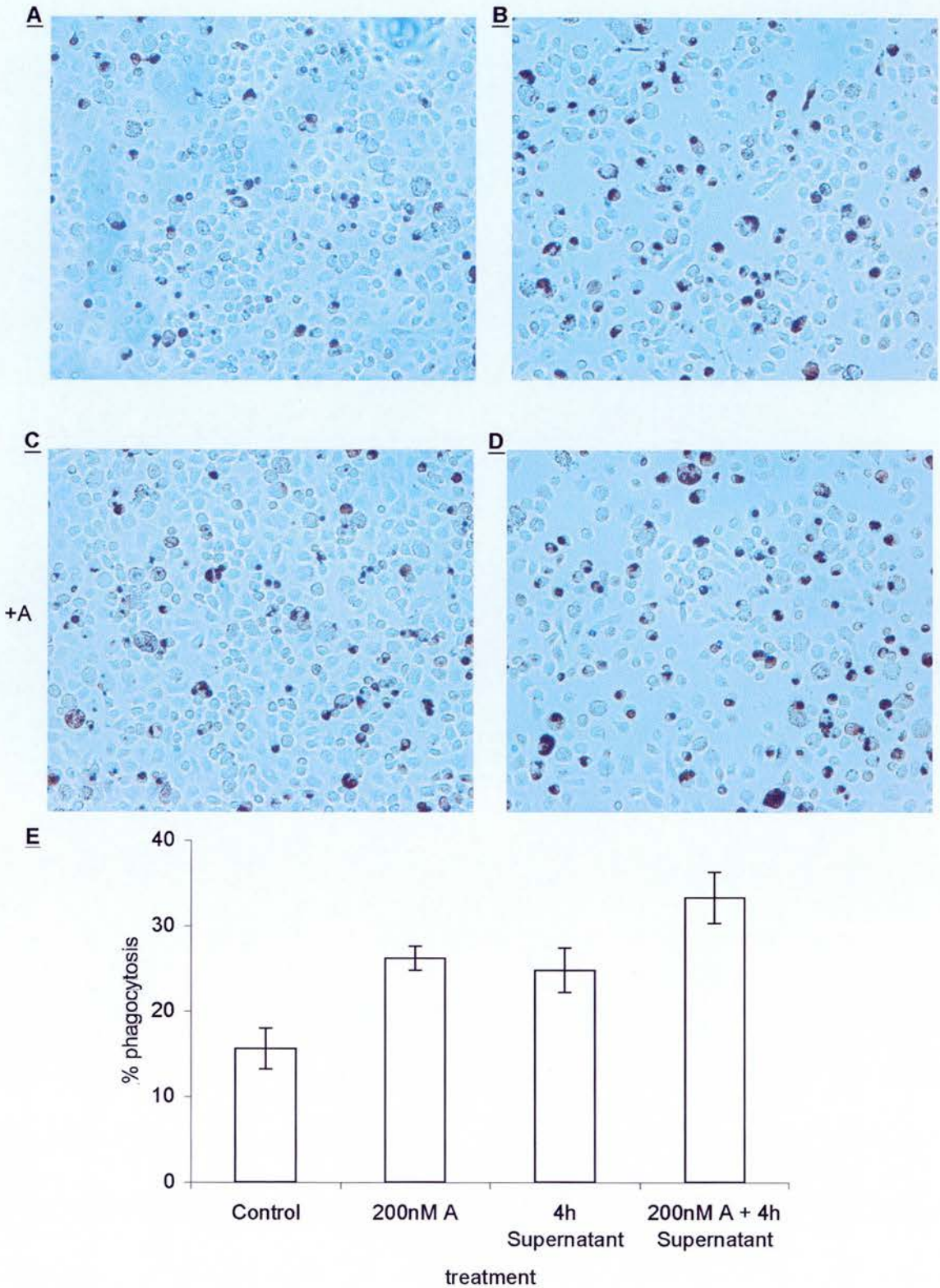

Control- 4h after thioglycollate
DT treated- 4h after thioglycollate

B


Control
DT treated
11β-HSD1 activity
% conversion A to B
100
80
60
40
20
0
4h
20h
time in culture

(previous page)

Figure 5-6: Monocyte depletion has no effect on the induction of 11β-HSD1 activity in TE peritoneal cells. Diphtheria toxin (DT) was administered to 'DTR' mice 24h prior to i.p thioglycollate treatment. Control mice were treated with thioglycollate alone. A, cytospins of peritoneal lavages showing elicited PMNs and monocytes from thioglycollate treated control mice, and depleted numbers of monocytes in washes from DT-treated mice. B, TE cells harvested at 4h were cultured and 11β-reductase activity measured in 10^6 cells measured over 20h. 11β-HSD1 activity is reported as % conversion of 200nM A to B. No significant difference in activity was seen in samples taken from monocyte depleted cultures compared to control at either 4h or 20h after plating. Photographs representative of 2 different mice of each group, and values are Mean +/- range of conversion levels of 2 mice of each group. Significance was not established.


A
B
C
+A
D
E
40
30
20
10
0
% phagocytosis
Control
200nM A
4h Supernatant
200nM A + 4h Supernatant
treatment

(previous page)

Figure 5-7: Phagocytosis of aPMNs by TEP Mϕs is augmented by inflammatory supernatant. 10^6 3d TEP Mϕs were incubated with 200nM A and/ or 50μl inflammatory supernatant for 24h, and then a 30min phagocytosis assay carried out with aged PMNs. A, control cells, no addition. B, addition of inflammatory supernatant alone. C, addition of A alone. D, addition of inflammatory supernatant and A.
E, addition of supernatant or A alone augmented phagocytosis over control levels. When combined, phagocytosis was increased approximately 2-fold over control levels. Photographs are representative of phagocytosis levels, and values are mean +/- range of counts of at least 600 TEP Mϕ from 2 separate expts carried out in duplicate. Due to difficulty of maintaining sterility during collection of supernatant it was only possible to successfully carry out this experiment twice, so significance was not established.

supernatant it was only possible to successfully carry out this experiment twice, so significance was not established.

5.2-4 IL-4 treatment up-regulates macrophage 11β-HSD1 activity, but does not itself promote phagocytosis

In the human model, IL-4, an anti-inflammatory cytokine has also been shown to up-regulate Mϕ 11β-HSD1 expression (Thieringer, Le Grand et al. 2001). Therapeutically there is more rationale to modulate 11β-reductase activity for the promotion of phagocytosis with anti-inflammatory rather than pro-inflammatory cytokines. Therefore, it was of interest to determine if the kinetic observations of Thieringer et al had a functional consequence for phagocytosis.

Initially, a range of IL-4 concentrations were used to establish effect on MD Mϕ 11β-reductase activity. The addition of 50ng/ml IL-4 proved detrimental to MD Mϕs, particularly when added to monocytes, causing rapid loss of morphology and detachment of cells (data not shown). Addition of lower concentrations of IL-4, (0.1-10ng/ml) to monocyte cultures on d1 caused induction of 11β-HSD1 activity above control levels within 24h (Fig.5-8A). At 48h, 200nM was completely converted in MD Mϕs treated with 1 and 10ng/ml IL-4, and 3-fold greater after 0.1ng/ml treatment, than control levels (Fig.5-8A). Similar trends were seen when IL-4 was given on d2 (Fig.5-8B), but when given on d3 the difference in induction over the next 48h was less pronounced because un-stimulated MD Mϕs were also strongly expressing 11β-HSD1 (Fig.5-8C).

Therefore these studies suggest that 11β-HSD1 was up-regulated in MD Mϕ by IL-4 with maximum effect at 1ng/ml. Unfortunately from this data it was not possible to determine whether the time-point at which 11β-HSD1 was first expressed was also accelerated.

To address the functional significance of this, it was of interest to discover whether the ability of IL-4 to up-regulate 11β-HSD1 was accompanied by an augmentation of phagocytosis in the presence of A. MD Mϕs were treated with 10ng/ml IL-4 and/ or

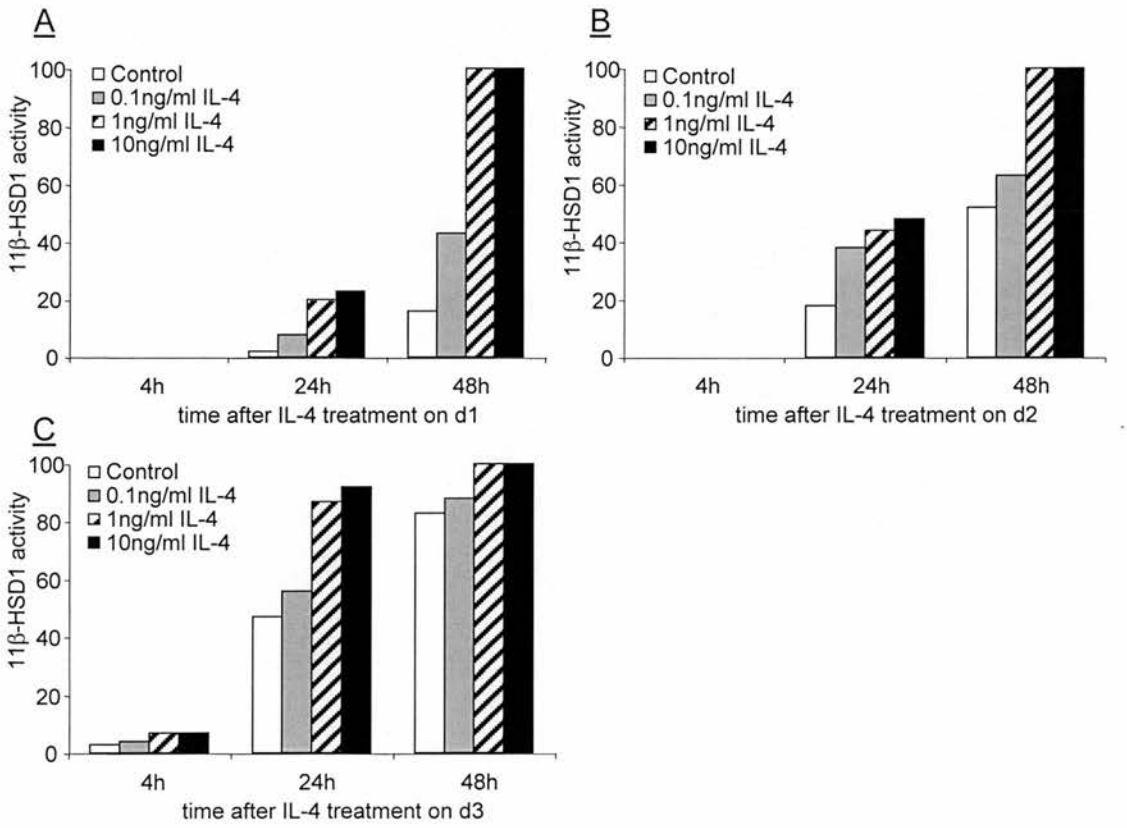


Figure 5-8: IL-4 treatment up-regulates 11β-HSD1 activity by human MD Mϕs. $4X10^6$ monocytes were differentiated and bioassayed for 11β-HSD1 activity (% conversion 200nM A to B). 1μM A and various doses of IL-4 (0-10ng/ml) were added to monocyte cultures on d1, d2 or d3 of differentiation. 4h, 24h and 48h later 11β-reductase activity was measured. A, control Mϕs (A only) treated on d1 had levels of 11β-reductase activity that were barely detectable at 24h, increasing to 16% conversion by 48h. In contrast, 11β-reductase activity at the same time-points was up-regulated by co-incubation with IL-4. The effect of IL-4 upon 11β-HSD1 activity in cells which have been differentiated for 2d or 3d was similar (B and C). Maximal effect was seen with 1ng/ml IL-4. Values shown are Means from 1 experiment carried out in duplicate. Significance was not established.

1μM A at either d0 or d2 of differentiation, and assayed for phagocytosis ability at d4. Control groups treated with A alone, at d2 (to coincide with 11β-HSD1 induction) showed increased phagocytosis over that of groups treated with A at d0 (Figs.5-9A and C). However cells treated with both IL-4 and A exhibited distinct morphological changes characteristic of giant cells, as well as cell loss and reduced levels of phagocytosis (Figs.5-9B and D). The earlier the exposure to IL-4, the more severe the morphological change (Fig.5-9B). This was an IL-4 specific effect and not an effect of GC exposure since cells exposed to IL-4 alone show identical changes (data not shown).

Therefore, IL-4 was shown to induce 11β-HSD1 activity in MD Mϕs. However, increased rates of A to B conversion did not augment phagocytic capacity, probably because of morphological changes to MD Mϕs caused by IL-4 treatment. Giant cells are poorly phagocytic for apoptotic cells (J Savill, personal communication). Time did not allow it possible to establish if similar changes were caused by 1ng/ml IL-4.

5.3 DISCUSSION

Following i.p injection of thioglycollate, 11β-HSD1 was dramatically up-regulated in cells resident to, or recruited to, the peritoneal cavity. This induction was rapid, with ≥12-fold increase in conversion for A to B detected at the first time-point (4h culture of peritoneal cells harvested 4h after thioglycollate injection), with 100% conversion of 200nM A achieved easily within 24h. This was followed with a decline to normal (resident) levels between d3 and d5. These data are in marked contrast to the timing of 11β-HSD1 induction during the *in vitro* differentiation of human monocytes. The rapid induction of 11β-HSD1 in response to thioglycollate is unlikely to be a consequence of differentiation and is probably mediated by the pro-inflammatory environment. In support of this, peritoneal supernatant taken 4h after thioglycollate injection induced 11β–HSD1 activity in RP Mϕs and WBC. However LPS, thioglycollate or non-inflammatory peritoneal supernatant (taken at d4) had no effect when added directly to the cells, suggesting that 11β-HSD1 activity was increased by an endogenous

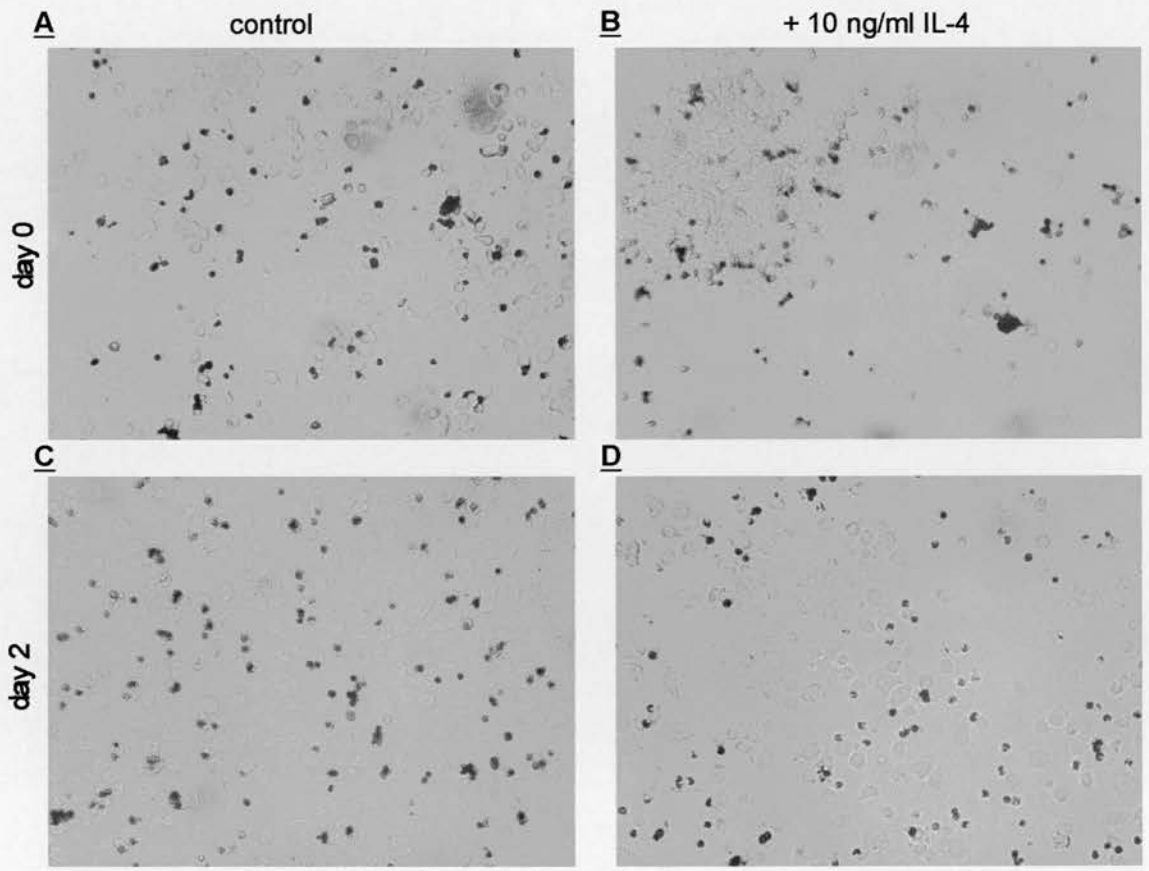


Figure 5-9: IL-4 treatment changes MD Mϕ morphology and does not augment phagocytosis of aPMNs. Human monocytes isolated from peripheral blood were differentiated into MD Mϕs, and 1μM A added at either d0 or d2. In addition, IL-4 (10ng/ml) was added at either d0 or d2 (B and D). Phagocytosis assays were carried out on d4 and ingested aPMNs stained with MPO. The photographs show that A augmented phagocytosis approximately 2-fold when added to d2 MD Mϕs (C), but not d0 monocytes (A). IL-4 addition inhibited phagocytosis to below control levels when added at either d0 (B) or d2 (D) and resulted in the formation of giant cell complexes. Monocytes were more susceptible to this morphological change than MD Mϕs (B and D). Images representative of 3 different experiments.

inflammatory mediator rather than directly by the inflammatory stimulus. This mediator is unknown but by virtue of the rapid up-regulation of 11β–HSD1 activity early in the inflammatory response, it is more likely to be a pro-inflammatory factor (or factors) rather than the Th2 cytokines reported to increase 11β–HSD1 expression during the differentiation of human monocytes *in vitro* (Thieringer, Le Grand et al. 2001).

One obvious candidate for mediating this effect is TNFα, which has been shown to increase 11β-HSD1 activity both in MC and adipocytes (Escher, Galli et al. 1997; Tomlinson, Moore et al. 2001). TNFα was detectable in diluted peritoneal supernatant 4h and 1d after induction of peritonitis. Clearly it will be more informative to repeat these experiments using undiluted supernatants. The other active constituents of this pro-inflammatory supernatant are as yet unknown and it will be of interest to identify potential modulators within this mileu. The ability to generate GC so early during an immune response suggests that the function of 11β-HSD1 may not only be to promote phagocytic function but also to modulate its pro-inflammatory responses. This data shows that synergy may exist between the pro-inflammatory activation of the HPA axis leading to increased release of adrenal-GC into the circulation (Mandrup-Poulsen, Nerup et al. 1995), and the induction of 11β-HSD1 activity in activated immune cells leading to increased GC in the tissues.

Data presented in Chapter 3 show that all Mϕs studied exhibit 11β-HSD1 activity. During the latter stages of peritonitis TEP Mϕs were the predominant cell type present, and it is most likely that, at these stages at least, they account for the 11β-reductase activity detected. However, it is not clear whether this reduction in activity following d3 is due to a down-regulation of TEP Mϕ 11β-HSD1 activity, or to the disappearance of another population of high 11β-HSD1 expressing cells. To address this problem, a number of experiments were carried out to distinguish the contribution of monocytes and PMNs to this induction, however, the results were somewhat contradictory. Firstly, when cells, collected 4h after induction of thioglycollate, were plated and subsequently washed, the majority of the 11β-reductase activity was retained in the adherent cultures (data not shown). This therefore suggests that the adherent monocytes/ Mϕs were

responsible. However, upon eosin and haematoxylin staining and examination by microscope it was apparent that the population was heterogenous and that activated granulocytes had unexpected adherence properties (data not shown).

Secondly, both cells within the WBC population and RP Mϕ population increased 11β-reductase activity in response to the addition of inflammatory supernatant. This data suggests that 11β-HSD1 was induced by an endogenous agent, since no induction was observed with thioglycollate treatment alone. Should cells within the WBC population be contributory to the induction of 11β-reductase, this suggests that 11β-HSD1 may be induced directly and not be dependent on transmigration across activated endothelial or mesothelial layers. However, the roles of resident Mϕs are somewhat ill-defined and whilst a primary function is to protect against spontaneous bacterial leakage from the gut, it remains to be seen whether they might also act as sentinels to co-ordinate the influx and activation of immune cells. RP Mϕ induction of 11β-HSD1 in the peritoneum may be a means of priming infiltrating monocytes for phagocytosis, regulating PMN apoptosis or controlling infiltration of leukocytes (Schleimer 1993; Meagher, Cousin et al. 1996; Giles, Ross et al. 2001). There are however, reports that RP Mϕ emigrate from inflamed sites during the initial stage, to return when resolution is complete and therefore may play a very limited role in the progression of inflammation (Melnicoff, Horan et al. 1989).

Thirdly, cell sorting on the basis of F4/80 and GR-1 was unsuccessful – ultimately, because the sorting process irreparably damaged the cells. This was contrary to expectation based on CIR experience in handling human blood-derived cells; thus the problem might relate to murine or inflammatory cell origins. However, it is believed that the monocytes were not sufficiently differentiated at 4h to be distinguishable from PMNs by GR-1 population, nor did monocyte F4/80 expression appear to be strong. Therefore, should the protocol be developed such that cells remain viable, other monocyte markers such as Mac-1 should included so that monocytes can be sorted on the basis of Mac-1 (high), GR-1 (low) double staining (Lagasse and Weissman 1996).

Finally, an attempt was made to deplete the circulating monocytes by DT treatment of DTR mice prior to induction of peritonitis. Although monocytes were not completely eliminated, no differences in 11β-reductase were seen between control and depleted groups, thus suggesting that monocytes do not contribute to the intra-peritoneal 11β-reductase levels, whereas RP Mϕs and/ or PMNs might.

Therefore, at this time, this important issue remains unresolved. Whilst murine Mϕs have both a constitutive and inducible 11β-HSD1 activity, it is very possible that activated PMNs exhibit activity as a way of regulating their own inflammatory functions and apoptosis.

Other approaches, should time have permitted, would have been either (i) to localise 11β-HSD1 mRNA transcripts in specific peritoneal inflammatory cells by in situ hybridisation analysis, (ii) isolate mononuclear cells and granulocytes from peritoneal lavages via an adaptation of the discontinuous Percoll gradient method for isolating human leukocytes, or (iii) separate the monocytes, RP Mϕs and PMNs from a mixed population by passing the cells through columns of immuno-magnetic beads specific for each cell type.

In humans it is clear that monocytes, fresh PMNs and aged PMNs do not exhibit 11β-HSD1 activity under non-inflammatory conditions. 11β-HSD1 induction can be promoted in differentiating monocytes by IL-4 treatment, but in the culture system employed in this study the MD Mϕ phenotype changed and characteristic morphology and phagocytic capacity was lost. During the course of this work, Thieringer and colleagues published a study in which 11β-HSD1 induction in human monocytes was reported with IL-4 at a concentration of 50ng/ml (Thieringer, Le Grand et al. 2001)– a concentration, which in this thesis proved toxic to monocytes. No functional responses of monocytes or phagocytes were tested in the Thieringer study (Thieringer, Le Grand et al. 2001). It is unlikely that such an anti-inflammatory Th2 cytokine would be present in an inflammatory mileu early in the infection. Unfortunately, it was not possible to source

a human pro-inflammatory exudate to test the induction potential of monocytes or activated human PMNs, in the same way that murine cells were tested.

Finally, preliminary experiments suggest that phagocytosis of aPMNs by TEP Mϕs can be augmented by inflammatory supernatant. When combined with A treatment however, this supernatant augmented phagocytosis even further. It is to be deduced that the induction of 11β-HSD1 in an environment where substrate concentration is not limiting serves to increase the rate of B amplification and increases phagocytic capacity of Mϕs through concentration and time-dependent mechanisms.

At this stage, it is worth comparing how data from this thesis supports the two contrasting phagocyte 11β-HSD1 regulatory mechanisms recently published by Escher and Thieringer (Escher, Galli et al. 1997; Thieringer, Le Grand et al. 2001).

The 'Thieringer hypothesis' is that the curbing of an inflammatory response via anti-inflammatory cytokines (IL-4 and IL-13) and the generation of active GC (by 11β-HSD1 induction) would facilitate both immune suppression and increase Mϕ clearance capacity. In support of this is the finding that ingestion of apoptotic cells does not trigger pro-inflammatory mediator release, even when the Mϕ is subsequently challenged with LPS, but actually promotes the release of anti-inflammatory cytokines associated with the resolution phase such as TGF-β (Meagher, Savill et al. 1992; Fadok, Bratton et al. 1998). Therefore, a shift from Th1 towards Th2 cytokines during inflammation would up-regulate 11β-HSD1, augment phagocytosis and thus create a positive feedback mechanism until resolution was complete (Fig.5-10).

From the study by Escher et al, it can be hypothesised that MC were limiting the chance of inappropriate tissue damage, in an autocrine fashion, by curbing their own pro-inflammatory secretion through the production of suppressive GC. Furthermore, as with Mϕs, ingestion of apoptotic cells by MC is not accompanied MC by pro-inflammatory cytokine release (Hughes, Liu et al. 1997). Therefore, in this system, pro-inflammatory cytokines would induce 11β-HSD1 and the resulting amplification of B would augment clearance as well as suppress subsequent pro-inflammatory action. This might not be

counter-intuitive since Mϕ phagocytic capacity can be programmed by GC exposure early in its differentiation pathway (Giles, Ross et al. 2001). The down-regulation of pro-inflammatory mediators occurring after phagocytosis might act as a safety mechanism to further curb inappropriate cytokine release during the resolution phase (Fig.5-10). The data in this thesis supports Escher's work, in that it suggests (albeit indirectly) that pro-inflammatory mediators are most likely responsible for 11β-HSD1 up-regulation, in a fashion that does not appear to lead to Mϕ dysfunction or phenotypic change. It is of note therefore, that preliminary experiments detected TNFα present in peritoneal fluid 4h and 1d after onset or peritonitis. It will be of interest to identify other possible inducers within the inflammatory mileu.

Next, the consequences of 11β-HSD1-deficiency, *in vivo*, during TE peritonitis were examined.

5.4 SUMMARY

- 11β-HSD1 activity was up-regulated by peritoneal cells *in vivo* within the first 4h of peritonitis.
- 11β-HSD1 activity remained induced until d3 of peritonitis, and returned to control level once inflammation had resolved.
- Induction of 11β-HSD1 was mediated by endogenous factors rather than as a direct effect of inflammatory agents.
- It was not been possible to identify the cell types responsible for the dramatic up-regulation of 11β-HSD1 activity. The most likely candidates were resident peritoneal Mϕs or infiltrating monocytes - but it cannot be discounted that 11β-reductase induction is an, as yet, unrecognised property of the inflammatory PMN.
- Addition of inflammatory supernatant induced 11β-HSD1 activity, and when combined with A treatment further augmented phagocytic potential of the Mϕ.

- IL-4 was able to up-regulate 11β-HSD1 activity but did not promote a pro-phagocytic MD Mϕ phenotype, instead stimulating giant cell formation.

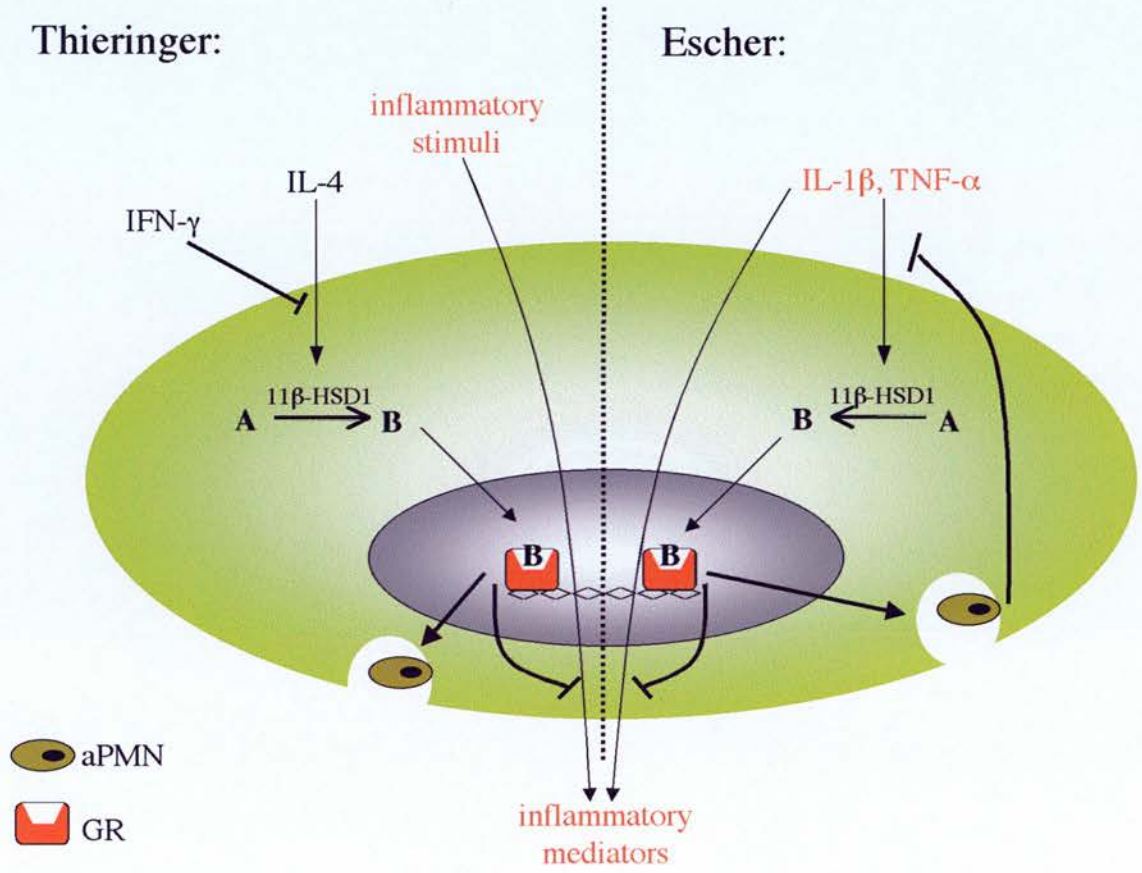


Figure 5-10: Diagram of proposed mechanisms of regulation of Mϕ 11β-HSD1 expression.

Chapter 6:

Consequences of 11β-HSD1 activity *in vivo*

6.1 INTRODUCTION

It has been shown in previous chapters that 11β-HSD1 is expressed in Mϕs and *in vitro*, inert A can augment phagocytosis of aPMNs – but not if the enzymic activity is inhibited. Furthermore, phagocytic capacity of TEP Mϕs for administered aPMNs can be increased by Dex treatment *in vivo*. The rapid induction of 11β-HSD1 during sterile peritonitis suggests that activity may play a physiological role in promoting the safe resolution of inflammation. To explore the importance of this *in vivo*, a mouse model was used in which the 11β-HSD1 gene has been disrupted by homologous recombination (Kotelevtsev, Holmes et al. 1997). The allele was subsequently crossed from the original 129/Ola strain to C57BL/6 strain by Dr. Janice Paterson, CIR.

It was important to determine whether differentiation of the 11β-HSD1-deficient Mϕ was different to WT, by examining basic Mϕ functions and competency to phagocytose aPMNs. Furthermore, it was also necessary to establish if 11β-HSD1-deficient Mϕs were GC-responsive to a similar degree as WT Mϕs. Then, the effects of 11β-HSD1-deficiency were examined during an acute inflammatory response, *in vivo*, by directly comparing the course of sterile peritonitis when induced in WT and 11β-HSD1-deficient mice. Using this model, inflammatory Mϕ maturation, clearance capacity and cytokine profile were also examined.

6.2 RESULTS

6.2-1 11β-HSD1 is required for augmentation of phagocytosis of apoptotic neutrophils by 11-dehydrocorticosterone

To investigate whether phagocytic responses were normal in 11β-HSD1-deficient Mϕs, BMD Mϕs were cultured for 7d in a non-inflammatory environment and assessed for phagocytic competency. Both genotypes of BMD Mϕs ingested saturating numbers of latex beads, suggesting that no intrinsic defect in phagocytosis was apparent in 11β-HSD1-deficient BMD Mϕs, compared to WT Mϕs (Fig.6-1).

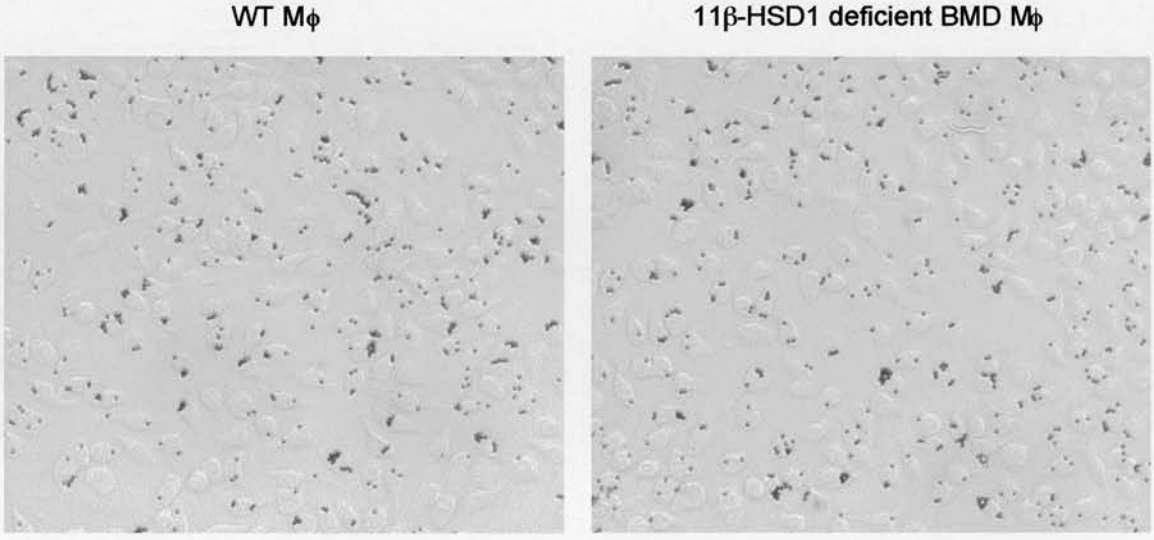


Figure 6-1: WT and 11β-HSD1-deficient BMD Mϕs ingest similar numbers of latex beads. A saturating number of latex beads (approx 5:1) were added to cultures of d7 WT BMD Mϕs or 11β-HSD1-deficient BMD Mϕs for 30 min. Both populations of Mϕ ingested high numbers of latex beads, and individual Mϕs were observed to ingest multiple beads. No differences between the genotypes were detected.

Next, to establish that Mϕ responses to B were normal in 11β-HSD1-deficient Mϕs, both WT and 11β-HSD1-deficient BMD Mϕs were incubated with concentrations of B or A within the physiological range for 48h and the effect upon phagocytosis measured.

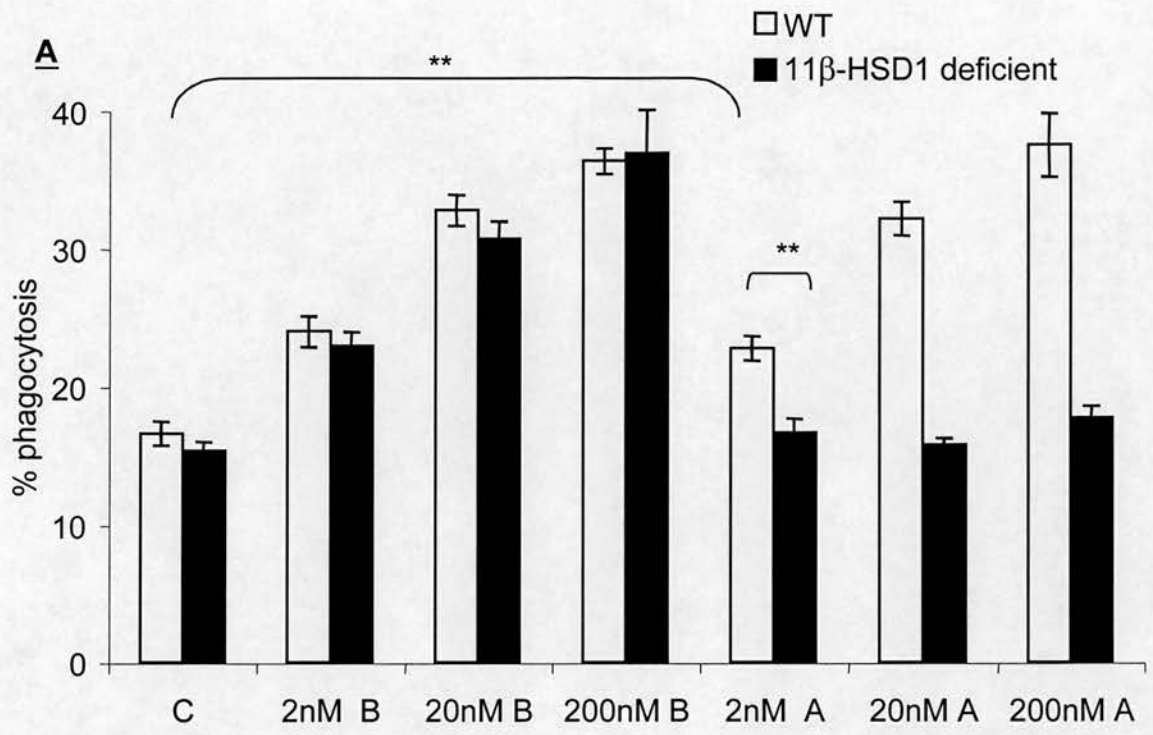
As expected, both B and A elicited a concentration dependent increase in phagocytosis by WT Mϕs over 48h, with significant effects seen even at low nM concentrations (Fig.6-2A). Phagocytosis by 11β-HSD1-deficient Mϕs was identically promoted by active B. However, A was completely without effect on phagocytosis by Mϕs from 11β-HSD1-deficient mice (Fig.6-2A). This confirmed that the augmentation of phagocytosis in response to A is due its re-activation to B by 11β-HSD1.

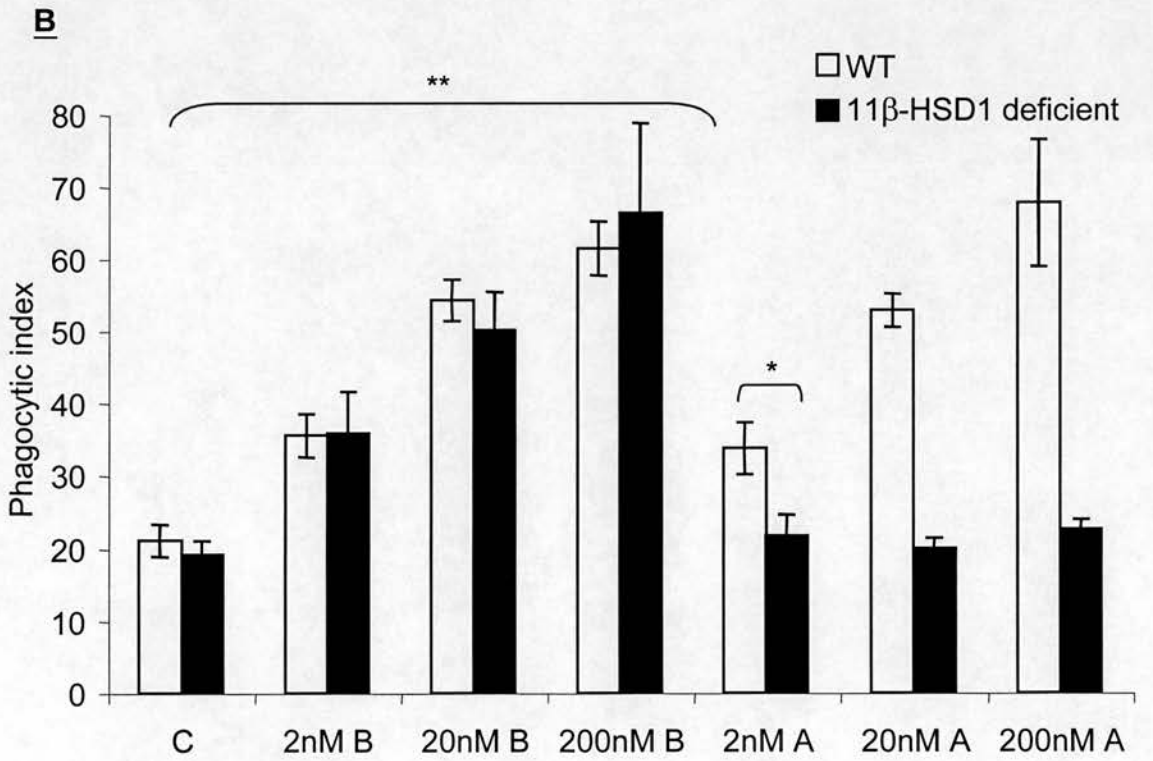
In addition to increasing phagocytosis, GCs also increased the ability of Mϕs to ingest multiple cells (the "phagocytic index" being the number of aPMNs ingested by 100 Mϕs (Giles, Ross et al. 2001)). B and A were similarly effective upon WT Mϕs, increasing phagocytic index 3-fold over control levels (Fig.6.2B). B was equally effective in augmenting the phagocytic index of 11β-HSD1-deficient Mϕs. In contrast, A was completely ineffective upon 11β-HSD1-deficient Mϕs (Fig.6.2B).

6.2-2 Time-course of sterile peritonitis in WT and 11β-HSD1-deficient mice

To establish the importance of local modulation of GC by 11β-HSD1 during an inflammatory response, the dynamics of TE peritonitis were compared in WT and 11β-HSD-deficient mice. Both genotypes of mice were injected i.p with 1ml 3% thioglycollate, and peritoneal cells harvested over the course of 4d. F4/80 (Mϕ) and GR-1 (PMN) positive cells in the peritoneal lavages were assessed by flow cytometry using CellQuest software (refer to section 2.2-10.3). Cytospins of these cells were also stained with eosin and haematoxylin for microscopic examination.

Appendix 1 shows flow cytometry data and cytospin images for mice of each genotype, studied over a 4d time-course of peritonitis. Briefly, peritoneal cells from healthy state animals (which had not been injected with thioglycollate) stained strongly for F4/80 by flow cytometry, consistent with a cell population predominately consisting of RP Mϕs


A
WT
11β-HSD1 deficient
**
**
% phagocytosis
0
10
20
30
40
C
2nM B
20nM B
200nM B
2nM A
20nM A
200nM A


B
WT
11β-HSD1 deficient
**
*
Phagocytic index
0
10
20
30
40
50
60
70
80
C
2nM B
20nM B
200nM B
2nM A
20nM A
200nM A

previous page)

Figure 6-2: 11β-HSD1-deficient Mϕs show normal augmentation of phagocytosis by B, but not A. BMD Mϕs were cultured for 7d, with A or B added at d5. On d7, aged PMNs were allowed to interact with the Mϕs (at a ratio of 4:1) for 30 min, and % of Mϕs that had ingested 1 or more a PMNs was assessed by microscopy.

A, WT Mϕs respond in a dose-dependent manner to both B and A. 11β-HSD1-deficient Mϕs, however, were similarly responsive to B, but not A. B, phagocytic index of ingested aPMNs per 100 Mϕs shows that A or B enhance the capacity of individual WT Mϕs to ingest multiple aPMNs, thereby further augmenting clearance potential. Only B was effective in increasing phagocytic index of 11β-HSD1-deficient Mϕs. Values shown are mean +/- SEM of counts of at least 600 Mϕs from 4 mice of each genotype, carried out in duplicate. *$P<0.05$, **$P<0.001$ (ANOVA), as indicated.

(Appendix 1, Figs.7-1 and 7-2). Also the absence of GR-1 positivity is consistent with negligible numbers of PMNs and monocytes being present. 4h after the onset of peritonitis, PMNs and monocytes were elicited into the peritoneum and the level of GR-1 staining increased accordingly. The level of F4/80 staining decreased at 4h. The reason for this was unclear since RP Mϕs were still visible on the cytospins (Appendix 1, Figs.7-3 and 7-4). However, F4/80 staining was increased 1d later, consistent with the maturation of inflammatory TEP Mϕs (Appendix 1, Figs.7-5 and 7-6). Over the next 2d (d3 and d4 of peritonitis), the level of GR-1 staining decreased and PMNs were deleted from the peritoneal cell population. By d4, only F4/80 staining was present, consistent with a cell population that was predominantly TEP Mϕs (Appendix 1, Figs.7-11 and 7-12).

By flow cytometry, no differences were seen between WT and 11β-HSD1-deficient mice at any time-point studied. However microscopic analysis of the cytospins revealed subtle differences in the deletion of PMNs and is described below (refer to sections 6.2-4 and 6.2-5).

6.2-3 Ratios of inflammatory cells during peritonitis

The flow cytometry analysis described in section 6.2-2, did not give a quantitative measure of the cell types present. It was of interest to discover whether 11β-HSD1-deficiency resulted in differences in the ratios of cell-types during the course of peritonitis. At least 2000 cells per cytospin were scored for each time-point, and the percentage of Mϕs, PMNs, aPMNs and aPMNs within Mϕs (ingested aPMNs) within the total population calculated (Table 6-1). No significant differences were seen in the proportions (within the total cell population) of WT Mϕs to 11β-HSD1-deficient Mϕs, or WT PMNs to 11β-HSD1-deficient PMNs at any time-point (Table 6-1). Similarly, there were no differences between other low number cell types such as eosinophils (data not shown).

The total numbers of cells harvested from the peritoneum on d2 of peritonitis were counted by haemocytometer. A significantly greater number of inflammatory cells were

	Genotype	Mϕs	PMNs
Control	WT	91.71% +/- 4.66	5.07% +/- 4.96
	11β-HSD1-deficient	88.98% +/- 1.56	3.16% +/- 2.63
4h	WT	27.93% +/- 4.03	67.12% +/- 2.26
	11β-HSD1-deficient	23.48% +/- 4.17	68.06% +/- 4.97
1d	WT	51.82% +/- 5.18	36.34% +/- 6.24
	11β-HSD1-deficient	53.04% +/- 4.2	32.74% +/- 5.13
2d	WT	59.55% +/- 10.01	16.82% +/- 5.85
	11β-HSD1-deficient	62.57% +/- 4.56	13.75% +/- 1.71
3d	WT	92.12% +/- 0.98	1.43% +/- 1.53
	11β-HSD1-deficient	89.56% +/- 2.56	0.40% +/- 0.37
4d	WT	97.10% +/- 1.82	0.09% +/- 0.08
	11β-HSD1-deficient	96.15% +/- 1.3	0.19% +/- 0.26

Table 6-1: Ratios of Mϕs and PMNs during TE peritonitis. Mϕs and PMNs were scored as a % of the total cells harvested in peritoneal lavages at specified time-points after onset of peritonitis. Values represent Mean +/- SEM of counts of at least 2000 cells from each of 4 mice per time-point. No significant differences between WT and 11β-HSD1-deficient mice were found.

present in the lavages from 11β-HSD1-deficient mice (Fig. 6-3). Interestingly no differences were seen in the proportions (within the total cell population) of WT Mϕs to 11β-HSD1-deficient Mϕs, or WT PMNs to 11β-HSD1-deficient PMNs (Table 6-1), and therefore the observation cannot be attributed to a condition such as neutrophilia.

6.2-4 Free apoptotic neutrophils are observed in 11β-HSD1-deficient mice at d2 and d3 of peritonitis

The number of free aPMNs present on each cytospin was scored and expressed as a % of total cells. 1d after the onset of peritonitis, significantly greater proportions of free apoptotic cells (mainly aPMNs) were observed in peritoneal lavages harvested from WT mice compared to 11β-HSD1-deficient mice (Fig.6-4). By d2 and 3 there was a greater than 2-fold reduction in the proportion of free aPMNs present in WT lavages, however, at these times there was approximately a 2-fold increase in free aPMNs present in 11β-HSD1-deficient lavages, compared to d1 (Fig.6-4).

6.2-5 11β-HSD1-deficient mice exhibit evidence of delayed clearance of apoptotic neutrophils during peritonitis

The number of Mϕs that contained ingested apoptotic cells on each cytospin was counted and expressed a % of total Mϕs. At d2 and d3 of peritonitis, greater proportions of Mϕs containing ingested apoptotic bodies were observed in 11β-HSD1-deficient cell populations than in WT populations (Fig.6-5). In WT mice, the vast majority of PMNs had been cleared by d2 (Table 6-1) - suggesting that ongoing clearance in 11β-HSD1-deficient mice at this time was abnormal (Fig.6-6).

These data show that 11β-HSD1-deficient Mϕs were able to ingest aPMNs, but the increased presence of ingested aPMNs inside the Mϕs at d2 and d3, as well as the higher level of free aPMNs suggested that the clearance process had been delayed or prolonged, possibly due to a defect in recognition, engulfment or degradation.

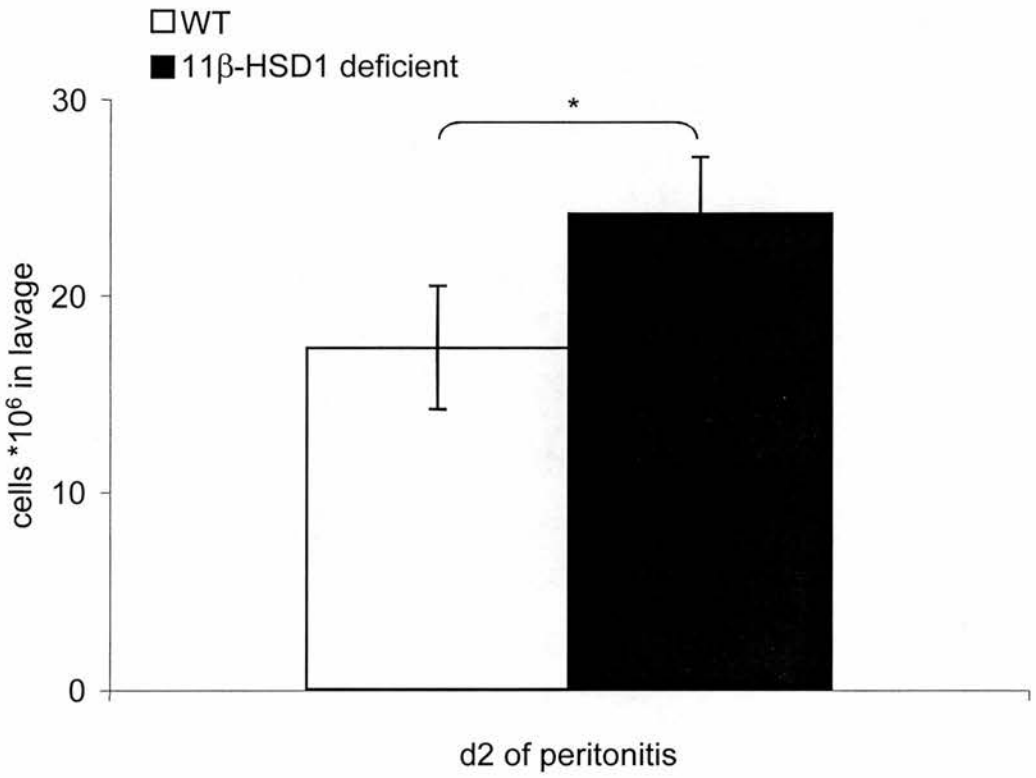


Figure 6-3: More inflammatory cells are present in lavages from 11β-HSD1-deficient mice than WT mice on d2 of peritonitis. The total number of cells lavaged from the peritoneums of mice on d2 of TE peritonitis were counted by haemocytometer. A significant increase in cell numbers was seen in lavages from 11β-HSD1-deficient animals. Values are Mean +/- SEM of haemocytometer counts of cells contained in 5ml of peritoneal lavage from each of 5 mice. *P<0.05 (t-test).

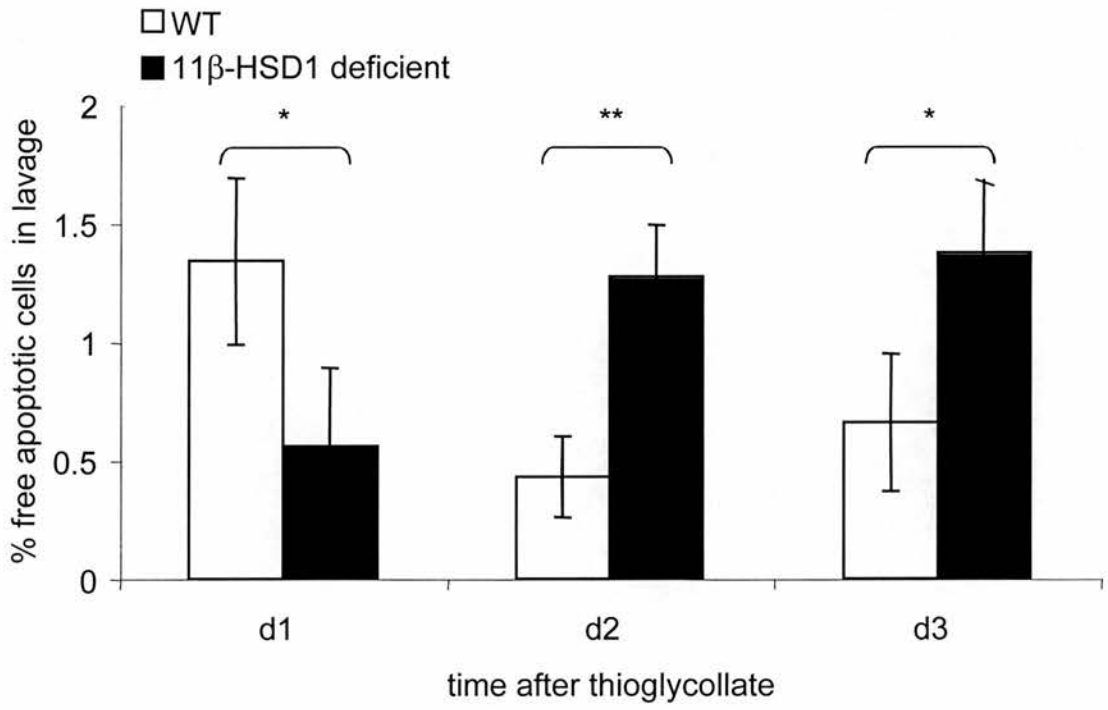


Figure 6-4: More free apoptotic cells are present in lavages from 11β-HSD1-deficient mice than WT mice on d2 and d3 of peritonitis. The proportion of free apoptotic cells was scored as a % of the total cells on each cytospin of peritoneal cells. WT mice had a greater % of free apoptotic cells at d1, whereas 11β-HSD1-deficient mice had a greater % at d2 and d3. Values represent Mean +/- SEM of counts of at least 2000 cells from each of 4 mice per time point. *P<0.05, **P<0.001 (ANOVA), as indicated.

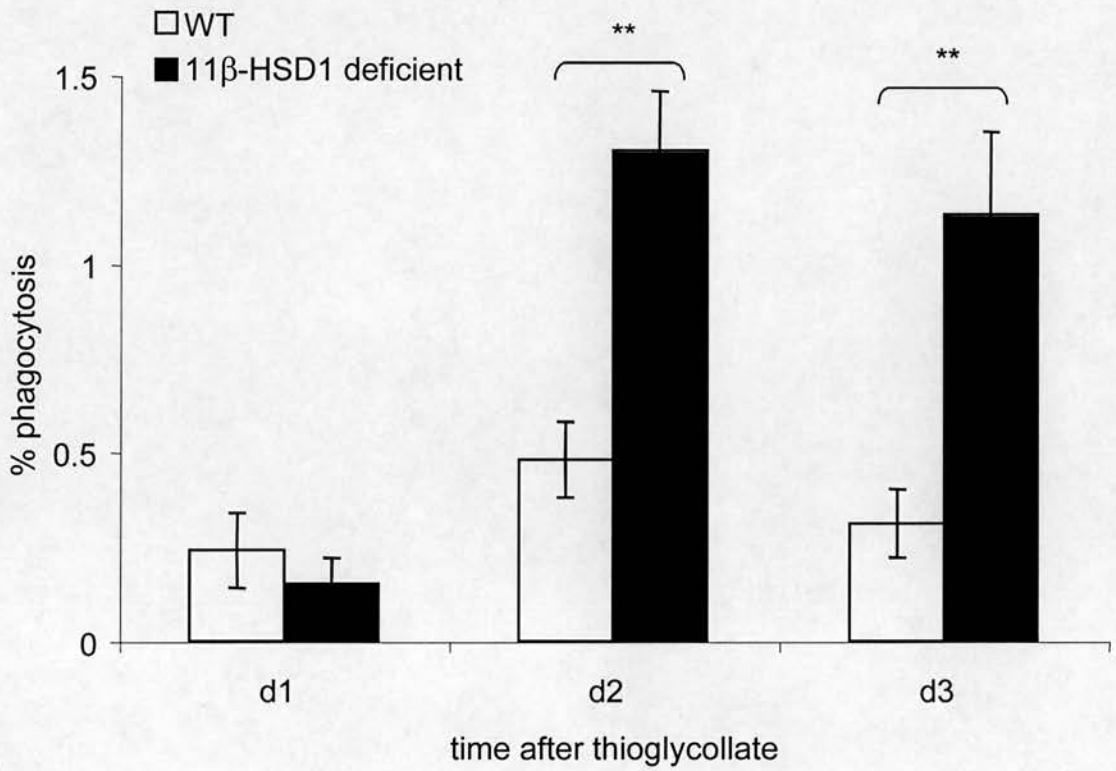


Figure 6-5: Greater levels of phagocytosis are observed by 11β-HSD1-deficient Mϕs than WT Mϕs on d2 and d3 of peritonitis. Phagocytosis was expressed as % of the total Mϕs in the peritoneal lavage that contained an apoptotic body. Greater phagocytosis was observed in Mϕs from 11β-HSD1-deficient mice compared to WT, at d2 and d3 of peritonitis. No significant differences were seen between the genotypes at d1. Values represent Mean +/- SEM of counts of at least 2000 cells from each of 4 mice per time point. **P<0.001 (ANOVA), as indicated.

Figure 6-6: Free apoptotic cells and ingested apoptotic cells are observed in lavages from 11β-HSD1-deficient mice 2d after onset of peritonitis. Representative image taken from cytospins of cells from a 11β-HSD1-deficient mouse during d2 TE of peritonitis. Note the free apoptotic cells (|) and the ingested apoptotic cells within the Mϕs (→).

6.2-6 11β-HSD1-deficient macrophages have a delay in achieving phagocytic competency *in vivo*

To investigate whether the delayed clearance of aPMNs in 11β-HSD1-deficient mice was due to a phagocytosis defect or a delay in achieving phagocytic competency, exogenous aPMNs were administered by i.p injection to both WT and 11β-HSD1-deficient mice at d1 and d2 of TE peritonitis (refer to section 2.2-10.2). Figure 6-7 shows that phagocytosis of aPMNs by WT TEP Mϕs at d1 was greater than by 11β-HSD1-deficient TEP Mϕs. However, at d2, the level of phagocytosis by WT Mϕs was reduced, but phagocytosis by 11β-HSD1-deficient Mϕs was significantly augmented to levels comparable to the WT Mϕ at d1 (Fig.6-7).

These data confirm that 11β-HSD1-deficient Mϕs had a delayed attainment of phagocytic capacity for apoptotic cells.

6.2-7 TNFα and nitric oxide levels during peritonitis

GCs modulate the transcription of numerous genes encoding inflammatory mediators (reviewed in section 1.2-3). It is possible that the local amplification of GC action within the peritoneum by 11β-HSD1 may also have immunomodulatory consequences. Expression of TNFα and iNOS (the inducible form of nitric oxide (NO)), both potent pro-inflammatory regulators, have been shown to be inhibited by GCs (Di Rosa, Radomski et al. 1990) (reviewed in (Barnes 1998)). Therefore, this part of the study attempted to address the question of whether a consequence of inability to amplify GC during peritonitis, would be the dysregulation of i.p levels of TNFα and NO.

Time only allowed preliminary experiments in which TNFα and nitrite levels in diluted supernatant (collected by centrifugation of peritoneal lavages) were measured by ELISA and Griess assay respectively (refer to section 2.11). Whilst TNFα was detected at 4h and 1d after the onset of peritonitis, no clear differences in TNFα levels were observed between peritoneal fluid from WT and 11β-HSD1-deficient (Fig.6-8). The Griess assay did not prove sensitive enough to detect nitrite levels at any time-points from either genotype of mouse (data not shown).

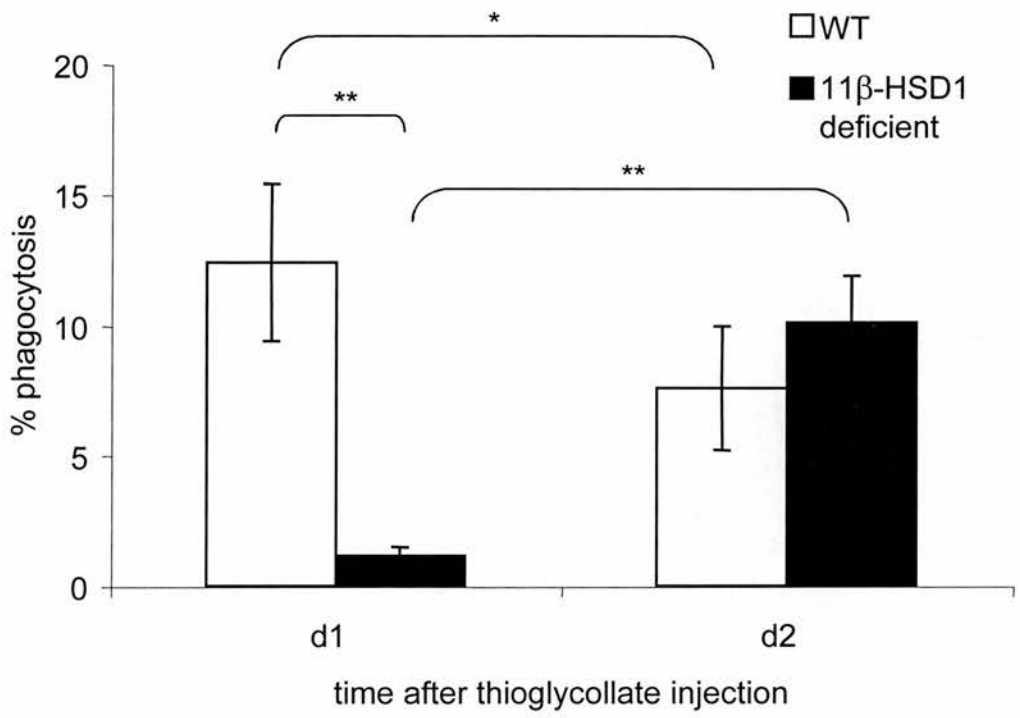


Figure 6-7: 11β-HSD1-deficient Mϕs have a delayed acquisition of phagocytic competency *in vivo*. Aged PMNs were administered by i.p injection at d1 and d2 after the onset of TE peritonitis. At d1, a significantly greater number of WT Mϕs, than 11β-HSD1-deficient Mϕs were able to ingest aPMNs. By d2, 11β-HSD1-deficient Mϕs had acquired the ability to ingest aPMNs. Values shown are Mean +/- SEM at least 500 cells counted by microscopy for each of 4 mice of each genotype. *P< 0.05, **P< 0.001 (ANOVA), as indicated.

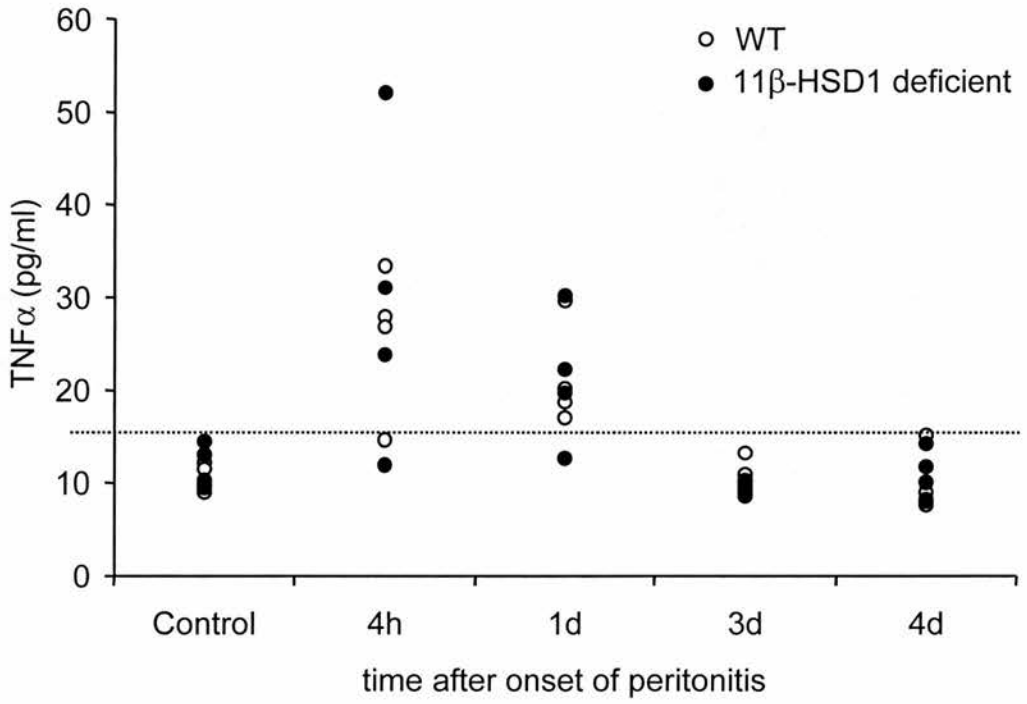


Figure 6.8: No differences in the i.p levels of TNFα are detected in peritoneal lavages from WT and 11β-HSD1-deficient mice. 3ml peritoneal supernatant was returned from the 4ml PBS bolus used to harvest peritoneal cells. Cells and debris were removed by centrifugation, and TNFα concentration was measured by ELISA. Even in diluted samples, TNFα was present at detectable levels in samples taken 4h and 1d after i.p thioglycollate injection. The dotted line indicates detection threshold of the ELISA kit.

6.3 DISCUSSION

The data presented in previous chapters show that 11β-HSD1 expression could potentially amplify GC action during an inflammatory event such as peritonitis. In order to address the possible role of 11β-HSD1 and its contribution via conversion of endogenous A to B during inflammation, peritonitis was induced in 11β-HSD1-deficient mice and subsequent events and resolution assessed.

The availability of 11β-HSD1-deficient mice allowed both the *in vitro*, and *in vivo*, maturation of Mϕs in the absence of 11β-HSD1 activity. The data suggest that 11β-HSD1 expression is not a pre-requisite for Mϕ differentiation and maturation to a phagocytic phenotype. *In vitro*, BMD Mϕs of both WT and 11β-HSD1-deficient genotypes ingested saturating numbers of latex beads, and untreated cells displayed a similar phagocytic index, thus demonstrating that, *in vitro* at least, non-specific and specific engulfment mechanism may be independent of 11β-HSD1.

Similarly, *in vitro* deficiency of 11β-HSD1 did not alter a Mϕ's responsiveness to active GC, and in fact, 11β-HSD1-deficient Mϕs were identical to WT in their GC-sensitivity as shown by similar phagocytic responses to B. However, 11β-HSD1-deficient Mϕs were completely unresponsive to A - confirmation again that the action of 11β-HSD1 is responsible for the re-activation and subsequent effect of A.

Therefore, in a non-inflammatory *in vitro* environment at least, Mϕ differentiation and GC-dependent pathways are not dependent on 11β-HSD1 induction. It is important to note that the effect of A on WT Mϕs was apparent with 48h exposure to concentrations as low as 2nM, which is well within the 1-5nM range of A concentration normally detected in plasma (Kotelevtsev, Holmes et al. 1997; Harris, Kotelevtsev et al. 2001). Tissue levels of A in WT mice are not known but they are probably of significance since recent data suggests that maintenance of B levels within tissues are largely dependent on the action of 11β-HSD1, in which A is the substrate (Yau, Noble et al. 2001).

GCs are known to inhibit leukocyte influx through suppression of chemo-attractants and endothelial-activating cytokines (Schleimer 1993). An intriguing observation made on

d2 of peritonitis was that during the initial stages of inflammation, 11β-HSD1-deficient mice recruited greater numbers of inflammatory leukocytes to the peritoneum than WT mice. This however, was not due to abnormal recruitment of any one cell-type, since the proportions of recruited leukocytes between the genotypes were very similar. This not only implies that the inability to regenerate GC in the peritoneum resulted in a prolonged period of cell infiltration, but also that the hyper-secretion of B seen in 11β-HSD1-deficient mice had no consequence in the peritoneal cavity. Studies by Yau et al suggest that WT mice are largely dependent on the action of 11β-HSD1 for intra-brain levels of B (Yau, Noble et al. 2001). Preliminary attempts were made to determine the i.p concentration of B during peritonitis using a B-specific radioimmunoassay (section 2.11-3). Unfortunately these were unsuccessful and it will be of great interest to measure levels of both B and A in the peritonea of WT and 11β-HSD1-deficient mice, and thus determine whether 11β-HSD1 action significantly contributes to B levels during peritonitis. It is possible to speculate that low i.p B levels might fail to suppress leukocyte secretion of pro-inflammatory cytokines such as TNFα. Detection of TNFα levels in peritoneal supernatants was possible, however no differences were seen between WT and 11β-HSD1-deficient mice in preliminary experiments.

Analysis of the cell surface expression of F4/80 by flow cytometry found no discernable difference between WT and 11β-HSD1 Mϕs at all time-points studied. F4/80 is a commonly used marker of Mϕ differentiation, but its expression may not be an indicator of function. In fact, it has been reported that Mϕ F4/80 surface expression decreases upon activation (Ezekowitz and Gordon 1982). This perhaps explains the reduction in F4/80 staining seen by flow cytometry, despite the continued presence of RP Mϕs in peritoneal populations recovered 4h after onset of peritonitis.

Similarly, no differences in the emergence and disappearance of GR-1 positivity, indicative of infiltration and clearance of PMNs was observed between WT and 11β-HSD1-deficient mice. In addition, both genotypes of mouse had fully resolved peritonitis by d4, such that the granulocyte population had all but disappeared. Therefore TE peritonitis was self-resolving in 11β-HSD1-deficient mice.

Phagocytosis and degradation of aPMNs, *in vivo*, is a rapid process and therefore evidence of a small number of phagocytic events in a 'snap-shot' of inflammation is most probably representative of substantial, on-going cell deletion (Savill 1997). In peritoneal lavages of 11β-HSD1-deficient mice collected on d2 and d3 of peritonitis, there was clear evidence of not only increased numbers of free apoptotic cells but also of significant numbers of Mϕs containing ingested apoptotic cells. In contrast, WT mice showed greater numbers of free apoptotic cells than 11β-HSD1-deficient mice on d1 of peritonitis. This suggested that 11β-HSD1-deficient mice may have delayed or deficient clearance of apoptotic cells.

Finally, the abnormality in clearance of apoptotic cells in 11β-HSD1-deficient mice appeared to be due to a delay in Mϕs attaining competency to ingest apoptotic cells (24h later than WT), rather than a defect in phagocytosis. TEP Mϕs from WT mice were capable of ingesting exogenously administered aPMNs 1d after induction of peritonitis, whereas TEP Mϕs from 11β-HSD1-deficient Mϕs attained this capacity by d2. Although it was not possible to determine whether the rate of degradation of phagocytosed aPMNs was normal, clearly 11β-HSD1-deficient TEP Mϕ are able to phagocytose. In this experimental model of sterile peritonitis, the delay in phagocytosis did not compromise the resolution of TE peritonitis, however, it is possible that such a delay may be costly in conditions of neutrophilia, such as sepsis.

It is unknown whether the rate of constitutive apoptosis of granulocytes, *in vivo*, is affected by GCs. It has been shown that pharmacological levels of GCs can increase the rate of apoptosis of human eosinophils and delay that of human PMNs *in vitro* (Meagher, Cousin et al. 1996). However, PMNs have been shown to express considerably less GR than other leukocytes and are therefore less responsive to physiological concentrations of GC than other leukocytes (Miller, Spencer et al. 1998). Also, the rate at which aging PMNs become apoptotic, *in vitro*, is delayed as the cell density increases (Hannah, Nadra et al. 1998), and therefore, with regard to the observations in 11β-HSD1-deficient mice, it cannot be discounted that either increased plasma B levels, or the increased i.p cellular density have the potential to affect the rate

of granulocyte apoptosis. Whilst no differences were observed in the proportions of PMNs present in WT or 11β-HSD1-deficient mice at any time-point during peritonitis, it is not possible to discount whether subtle differences in the apoptotic programme of 11β-HSD1-deficient PMNs would account for the phenotype observed, and further investigation is warranted.

6.4 SUMMARY

- In a non-inflammatory *in vitro* environment, 11β-HSD1 BMD Mϕs were fully GC-responsive but, unlike WT Mϕs, phagocytic mechanisms were unresponsive to the inert GC, A. Furthermore, no basic Mϕ phagocytic defect was observed between genotypes, judged by the capacity to ingest either apoptotic cells or latex beads.
- Both genotypes of mice fully resolved TE peritonitis within 4d.
- During peritonitis, greater numbers of inflammatory cells were elicited into the peritonea of 11β-HSD1-deficient mice, although no differences were apparent in the ratios of the peritoneal cells types present between WT and 11β-HSD1-deficient mice.
- 11β-HSD1-deficient mice appeared to have delayed clearance of aPMNs and/ or increased rates of PMN apoptosis. Peritoneal lavages taken at d1 from WT mice contained greater proportions of free aPMNs than lavages taken from 11β-HSD1-deficient mice. In contrast, lavages taken at d2 and d3 from 11β-HSD1-deficient mice contained greater proportions of free aPMNs than lavages taken from WT mice, as well as greater proportions of TEP Mϕs that show evidence of aPMN ingestion.
- No gross differences between WT and 11β-HSD1 TEP Mϕs were seen using the maturation marker F4/80, but acquisition of phagocytic competency by inflammatory TEP Mϕs was delayed by 24h in 11β-HSD1-deficient mice.

Chapter 7:

Discussion

7.1 SUMMARY

The significance of the apoptotic cell and its swift, non-inflammatory deletion by the Mϕ, has been defined by others, both *in vitro* and *in vivo* (reviewed in Chapter 1). Furthermore, new benefits of GC treatment have recently been suggested after the finding that pharmacological doses of GCs have regulatory effects upon Mϕ capacity for apoptotic cells (Liu, Cousin et al. 1999; Giles, Ross et al. 2001). Work contained within this thesis has, for the first time, demonstrated that local metabolism of physiological GCs at the inflammatory site may be important in determining the fate of apoptotic cells.

Briefly, it is shown that all phagocytes studied (human and murine) express 11β-HSD1 mRNA and the resulting enzyme activity is exclusively in the GC-reactivating, reductase direction. Human monocytes acquire this ability during differentiation. *In vitro*, the functional consequence of this is that inert A is converted to active B, which augments the phagocyte's capacity to ingest aPMNs. Upon induction of sterile peritonitis in mice, there is a dramatic up-regulation of 11β-HSD1 activity within the cells resident to, or recruited to, the peritoneal cavity. For peritonitis to resolve, apoptotic cells within the peritoneal cavity must be cleared by Mϕs. In 11β-HSD1-deficient mouse Mϕs, A fails to promote phagocytosis *in vitro* and, *in vivo*, phagocytic competency is attained as much as 24h later than in WT Mϕs. As a result, 11β-HSD1-deficient mice show a clearance deficiency of apoptotic cells during the resolution of peritonitis, suggesting a physiological role for 11β-HSD1 in promoting clearance of apoptotic cells.

There are great similarities between different inflammatory conditions and it is likely that 11β-HSD1 has a role in other classes of phagocyte. Preliminary *in vitro* data showed that 11β-HSD1 expression in MC can also increase phagocytosis in response to A, suggesting that "semi-professional" phagocytes may also have the potential via 11β-HSD1 to regulate their phagocytic capacity in pathologies such as glomerulonephritis.

This work raises interesting questions about the importance of local modulation of GCs and with particular relevance to phagocytes, further investigations are warranted.

7.2 GENERAL DISCUSSION AND FUTURE STUDIES

7.2-1 What factors regulate macrophage 11β-HSD1 expression?

Evidence from this thesis supports that of Escher et al, and Tomlinson et al, who reported 11β-HSD1 up-regulation by pro-inflammatory cytokines in MC and adipocytes, respectively (Escher, Galli et al. 1997; Tomlinson, Moore et al. 2001). Furthermore, not only did the effects of peritoneal pro-inflammatory supernatant induce 11β-reductase expression in cells resident in, or recruited to, the peritoneum, it also augmented phagocytic capacity (Ren et al have also reported that pro-inflammatory cytokines augment the phagocytosis of aPMNs by MD Mϕs (Ren and Savill 1995)). It seems unlikely that pro-inflammatory cytokine-based treatments hold therapeutic potential, but it is intriguing that pro-inflammatory stimuli should confer beneficial effects to a process important in the successful resolution of an inflammatory response. A related concept is that this may be an example of early events, previously thought to be exclusively involved in initiation and amplification of an inflammatory response, "programming" the inflammatory response for successful resolution later on. Thus it is possible that the early and rapid amplification of GC within inflamed tissue serves to control the extent of the physiological (and sometimes detrimental) immune response, whilst priming the Mϕ for a timely phagocytic response. It is also conceivable that the local generation of GC by phagocytes co-ordinates the action and lifespan of their 'prey'.

Evidence is also presented to suggest that an anti-inflammatory cytokine, IL-4, can regulate 11β-HSD1 expression, although an increased phagocytic phenotype was not conferred. Should 11β-HSD1 induction not be merely a consequence of differentiation, then this raises the possibility that the nature of the 11β-HSD1 induction may influence the differentiation and phenotype of the Mϕ. Indeed, this idea is consistent with both 11β-HSD1-deficient Mϕs, and C/EBP-ε-deficient Mϕs being viable, yet dysfunctional. It will be of interest to determine which, if any, exogenously added cytokines regulate the expression of 11β-HSD1 and whether differences in Mϕ functionality are associated.

In vitro, human MD Mϕs express 11β-HSD1 by d2 of culture. It is unknown whether induction occurs rapidly during inflammation, as it does in the murine system. Again, the use of cytokines or indeed human pro-inflammatory supernatant (if available) would help determine the responsiveness of 11β-HSD1 in human cells. The work of Giles et al has obvious parallels to this discussion (Giles, Ross et al. 2001). They showed that early exposure to GC altered differentiation of human monocytes resulting in a pro-phagocytic phenotype. Although a comparison across species, if a similarly rapid induction of monocyte 11β-HSD1 occurs in response to an inflammatory stimulus during human inflammation (as seen during murine peritonitis), it may deliver an early, pro-phagocytic phenotype-determining "dose" of physiological GC to the differentiating monocyte – particularly within tissues where "free" B levels may be substantially less than in plasma.

During peritonitis, 11β-HSD1 activity decreased from d3 onwards, to a level comparable to that prior to inflammation. Whilst it is possible that this is due to the gradual deletion of a small population of cells strongly expressing 11β-HSD1, it most likely reflects decreased 11β-reductase activity in the TEP Mϕ population - particularly since TEP Mϕs are by far the most predominant cell type by that stage. Two possibilities exist; (i) a down-regulation of 11β-HSD1 by TEP Mϕs occurs in response to the clearance of apoptotic cells, or (ii) the emigration of a sub-population of TEP Mϕs that have a strongly induced 11β-HSD1.

Further experiments will include analysis of 11β-reductase activity in 'fed' and 'non-fed' cultures of Mϕs. It will also be of interest to determine whether 11β-HSD1 activity is down-regulated differently in response to different classes of 'feed', such as aPMNs, thymocytes, latex beads, opsonized zymosan etc. Also, if a down-regulation of 11β-HSD1 in TEP Mϕs is confirmed, it would be informative to discover whether this is reciprocated in a reduction in GC-sensitivity by determining GR levels.

A more challenging idea to address is that of TEP Mϕ emigration. Mϕs emigrate to the lymphatics after inflammation has resolved (Bellingan, Caldwell et al. 1996). Direct

comparisons of 11β-reductase activity between Mϕs recovered from these structures and those still in the peritoneum on, for example, d4 of peritonitis should be attempted.

A reduction in GC amplification, or a reduction in GC-sensitivity once the inflammation has resolved is likely to be beneficial in avoiding inappropriately prolonged exposure to GCs.

7.2-2 11β-HSD1 and chronic inflammation

Chronic arthritis has been shown to up-regulate hippocampal 11β-HSD1 activity, but not heptatic 11β-HSD1 activity (Low, Moisan et al. 1994). No data exist as yet on 11β-HSD1 activity in Mϕs during chronic inflammation.

The factors controlling the progression from acute infection to chronic inflammation are not yet elucidated, but there is increasing evidence that the failure to clear apoptotic cells leads to necrosis and exacerbation of the response (reviewed in Chapter 1). In fact there is evidence that reduced numbers of phagocytic Mϕs that contain ingested apoptotic cells are recovered from the synovial fluid of rheumatoid arthritic patients, than from patients with acute reactive arthritis (Jones, Denton et al. 1993). This therefore suggests that phagocytosis is either down-regulated or inhibited. Furthermore, Mϕs have been shown to undergo a phenotypic change after phagocytosis in which they fail to release pro-inflammatory cytokines upon subsequent inflammatory challenge (Voll, Herrmann et al. 1997), and have a reduced ability to engulf apoptotic cells during subsequent phagocytosis assays (Erwig, Gordon et al. 1999).

By failing to respond to an inflammatory stimulus, it is possible that low or down-regulated Mϕ 11β-HSD1 activity is either a causative factor, or a consequence of chronic inflammation. It will be of interest to compare 11β-HSD1 expression and phagocytic abilities of Mϕs collected from chronically inflamed sites. A candidate model is that of rat arthritis, in which inflammation in different joints could be manipulated individually to represent normality, acute inflammation (eg. collagen) and chronic inflammation (eg. adjuvant-induced arthritis), and thus allow Mϕs to be compared directly, *ex-vivo*.

Finally, the ideal model to assess the role of 11β-HSD1 expression during chronic inflammation is the 11β-HSD1-deficient mouse. Its ability to resolve a number of acute challenges of varying degrees of severity, from repeated doses of i.p thioglycollate to resolving nephritis should be assessed.

7.2-3 The consequences of 11β-HSD1 deficiency

The inability to re-amplify B from inert A has identified a phenotype of delayed clearance of apoptotic cells in 11β-HSD1-deficient mice compared to WT. Clearly, in terms of a self-resolving peritonitis this deficiency has had no obvious detrimental effect. However, it will be interesting to determine whether the 11β-HSD1-deficient mouse can successfully resolve other types of inflammatory response (refer to section 7.2-2).

Another interesting phenotype that was observed was that of an increased inflammatory cell peritoneal infiltrate in the 11β-HSD1-deficent mice at d2 – the only time I was able to assess owing to limited animal supply. GCs can inhibit leukocyte influx (reviewed in (Schleimer 1993)), and whilst the mechanism behind this is not clear, it may be the case that the local amplification of GC by 11β-HSD1 action in tissues, rather than plasma GC, plays an important part in modulating the duration of inflammatory cell influx. Again, resolution of inflammation of a different severity, or within a different tissue or compartment could be compromised by increased cell load.

With regard to the plasma GC levels, it is unclear what influence circulating B levels have on intra-tissue B concentrations. Despite raised plasma levels in the 11β-HSD1-deficient mouse (refer to section 1.3-4), B levels, in the brain at least, are lower than WT, which suggests that tissues in the periphery may also be substantially dependent on 11β-HSD1 action for B (Yau, Noble et al. 2001). In the present study, it was not possible to determine peritoneal levels of B during peritonitis for either genotype of mouse. Therefore, it will be important to establish the concentrations of B and A during the evolution and resolution stages of peritonitis by radioimmunoassay or alternative analyses. In particular, if B levels are low in comparison to A during the initial stages of

peritonitis, this would imply that the generation of B from A by 11β-HSD1 is of physiological importance.

A future experiment that would, in part, address the contribution of the B-levels to the 11β-HSD1-deficient phenotype is that of adoptive transfer of 11β-HSD1-deficient monocytes to WT mice at various stages of peritonitis. Such cells would be harvested, stained with CM-Green and injected back into a WT mouse at an identical stage of peritonitis. By performing *in vivo* phagocytosis assays at different stages of peritonitis it would then be possible to determine if either 11β-HSD1 expression or B levels were key to the attainment of phagocytosis competency by comparing phagocytic abilities of WT and 11β-HSD1-deficient Mϕs. Preliminary experiments have shown that inflammatory cells taken from the peritonea of WT C57 BL/6 mice 1d after the onset of TE peritonitis, can be stained with CM-Green and successfully transferred to the peritonea of donor mice, within which labelled Mϕs differentiate to a normal phagocytic phenotype (unpublished observations).

Should the high plasma-B levels not be reflected within the peritoneum, then 11β-HSD1-deficient Mϕs might be expected to have a prolonged Th1 response in the absence of a GC-mediated switch to an anti-inflammatory Th2 profile. Analysis of peritoneal supernatant or culture medium of *ex-vivo* TEP Mϕs by ELISA may be sufficient to identify inappropriate cytokine secretion should it exist. Mature 11β-HSD1-deficient Mϕs appear to phagocytose normally, yet it will be important to determine whether they down-regulate pro-inflammatory secretion in response to phagocytosis of apoptotic cells in the same way as WT cells. Interestingly it was found that mice deficient for C/EBP-ε have a complete absence of IL-10 mRNA and activity (Tavor, Vuong et al. 2002) (refer to section 1.4-3).

It has been suggested that the deficient clearance of apoptotic cells, *in vivo*, may lead to the development of autoimmunity (Taylor, Carugati et al. 2000). It is, therefore, possible that the levels of auto-antibody present in the serum or kidneys of aged 11β-HSD1-

deficient mice may be higher than aged WT mice. Mice currently being aged for neurological studies will provide tissues for such a study.

Finally, an important, and unresolved issue is that of GR levels in the absence of 11β-HSD1. GR is often negatively regulated by GC levels, at least in the short term (Kitraki, Karandrea et al. 1999) (reviewed in 1.2-5). It is therefore likely that a 11β-HSD1-deficient Mϕ's sensitivity to GC will also be determined by an altered GR density. Analysis of GR mRNA levels in WT and KO TEP Mϕs by competitive RT-PCR are currently in progress.

7.2-4 Is modulation of 11β-HSD1 action a potential therapeutic target?

The efficacy of high dose, long-term, GC-based therapeutic regimes are compromised by side effects. It may therefore be desirable to target the beneficial effects of GC to phagocytes. This thesis has provided a novel therapeutic idea – that phagocyte 11β-HSD1 can be modulated such that the beneficial, phagocytic effects of GCs can be targeted locally to phagocytes using non-toxic concentrations of inert GC. Liposomes, injected systemically during arthritis, pass through the 'leaky' vasculature and are preferentially taken up by inflammatory Mϕs in the inflamed joint (personal communication from Dr Bart Metselaar, Utrecht). Therefore, it may be possible to target liposomes packaged with either (i), 11β-HSD1 inducers (if 11β-HSD1 expression is found to be down-regulated in chronic inflammation) or (ii), inert GCs, directly to the inflammatory foci. Inert GCs are preferable to active GCs because inappropriate leakage or delivery would only mediate effects on 11β-HSD1 expressing cells.

7.2-5 Would over-expression of 11β-HSD1 sensitise macrophages to the effects of A?

Murine TEP Mϕs are able to convert 200nM A to B over 24h, typically conferring a 2-fold augmentation of phagocytosis. 200nM was determined to be the most effective concentration for both A and B, implying that 11β-HSD1 is not the rate-limiting factor in mediating the effect of A. However, A was only equipotent to B over a treatment time of 48h. Therefore, to ask the question of whether an increased rate of conversion of A to

B is required to accelerate the availability of B (thereby enhancing the effect of B over a shorter period), defined numbers of 11β-HSD1 transgenes could be introduced to the Mϕs using Bacterial Artificial Chromosomes.

The murine data suggested that 11β-HSD1 expression was induced rapidly upon the induction of peritonitis. In contrast, 11β-HSD1 expression in human monocytes was constitutively induced during d2 of culture. Whether this is the case *in vivo*, or is an *in vitro* artefact in the absence of appropriate inflammatory signals remains to be determined. Nevertheless, it may still be of benefit to drive the expression of 11β-HSD1 in differentiating monocytes to promote the earlier acquisition, and greater capacity for phagocytosis. It may therefore be possible to use transgene technology to construct a monocyte-specific 11β-HSD1 transgene under the control of either the macrophage metalloelastase (MME) or the CD68 promoters. The MME promoter would induce 11β-HSD1 expression as the monocyte entered the inflamed tissue (Shapiro, Griffin et al. 1992), and the CD68 promoter would induce 11β-HSD1 expression in circulating monocytes (Li, Guidez et al. 1998). However, should it be confirmed that murine blood-borne monocytes already express 11β-HSD1, then this transgenic approach would serve to increase expression.

7.2-6 How do GCs potentiate phagocytosis?

It is of great interest to determine the down-stream mechanism by which 11β-HSD1 action may be augmenting phagocytosis. In the study by Liu et al, it was found that synthetic GC had no effect on the surface expression of components of the $\alpha_v\beta_3$ vitronectin receptor/thrombospondin/CD36 recognition mechanism, and had little effect on CD-14 (Liu, Cousin et al. 1999). Thus, GC action appeared to exert a 'pan-phagocyte' action in which different classes and lineages of phagocytes from different species were affected – some of which use distinct apoptotic cell receptor mechanisms. Recent work has established that members of the scavenger receptor family (putative apoptotic cell clearance receptors on human and murine Mϕs) are up-regulated on human monocytes during Dex treatment (Galon, Franchimont et al. 2002). It is therefore

possible that the earlier clearance of aPMNs achieved by WT Mϕs in comparison to 11β-HSD1-deficient Mϕs, is through a GC-inducible scavenger receptor expressed on the Mϕ surface as a consequence of the rapid induction of 11β-HSD1. Future work will determine the levels of scavenger receptor expression on WT and 11β-HSD1-deficient Mϕs in response to GC by Ab staining (eg. anti-2F8 for scavenger receptor A). Overall densities of surface expression could be assessed by flow cytometry, whereas receptor clustering could be assessed by fluorescence microscopy.

Evidence from human monocyte studies suggests re-organisation of cytoskeletal elements is necessary for GC-augmented phagocytosis (Giles, Ross et al. 2001). However, this was accomplished with pharmacological doses of Dex, in which Dex-treated monocytes adopted a distinct rounded morphology and were less adherent to plastic (Giles, Ross et al. 2001). This therefore may not be applicable to the present physiological murine system since WT Mϕs did not appear to differ in morphology to 11β-HSD1-deficient Mϕs. However Dex may be acting through 2 different mechanisms. In the *in vitro* culture of human MD Mϕs, Dex treatment for only 24h to monocytes was sufficient to effect the human monocyte to macrophage differentiation programme (Giles, Ross et al. 2001), whereas Dex addition for 24h to fully differentiated MD Mϕs did not change morphology, but increased phagocytic potential, possibly through the induction of a phagocytic receptor (Liu, Cousin et al. 1999).

7.2-7 Does 11β-HSD1 action affect other functions of macrophage biology?

11β-HSD-1-deficient mice have recently been reported to have altered lipoproteins, insulin sensitivity and triglycerides, and may therefore be less susceptible to atherosclerosis (Morton, Holmes et al. 2001). It is therefore very likely that Mϕ 11β-HSD1 biology will extend to new studies in which may for example, determine a link between 11β-HSD1, CD36 and lipid uptake.

7.3 CONCLUDING REMARKS

The work in this thesis has contributed to the fields of endocrinology and Mϕ biology. Most notably, it has identified a physiological role for 11β-HSD1 in promoting clearance of apoptotic cells.

Understanding the Mϕ-specific biology of 11β-HSD1 will increase our understanding of its role in the pathophysiology of inflammatory diseases. This may lead to novel therapeutic manipulations of steroid action.

Bibliography:

Adcock, I. M., Lane, S. J., Brown, C. R., Peters, M. J., Lee, T. H. and Barnes, P. J. (1995). "Differences in binding of glucocorticoid receptor to DNA in steroid-resistant asthma." J Immunol **154**(7): 3500-5.

Agarwal, A. K., Monder, C., Eckstein, B. and White, P. C. (1989). "Cloning and expression of rat cDNA encoding corticosteroid 11ß-dehydrogenase." J Biol Chem **264**: 18939-18943.

Agarwal, A. K., Mune, T., Monder, C. and White, P. C. (1994). "NAD+-dependent isoform of 11ß-hydroxysteroid dehydrogenase." J Biol Chem **269**: 25959-25962.

Albiston, A. L., Obeyesekere, V. R., Smith, R. E. and Krozowski, Z. S. (1994). "Cloning and tissue distribution of the human 11ß-hydroxysteroid dehydrogenase type 2 enzyme." Mol Cell Endocrinol **105**(2): R11-R17.

Alvarez, J., Surs, W., Leung, D. Y., Ikle, D., Gelfand, E. W. and Szefler, S. J. (1992). "Steroid-resistant asthma: immunologic and pharmacologic features." J Allergy Clin Immunol **89**(3): 714-21.

Amelung D, H. H. J., Roka L, Meyerheim G (1953). "Conversion of cortisone to compound F." J Clin Endocrinol Metab **13**: 1125-1128.

Andrews, M. J. (1998). "Leucocyte kinetics in experimental peritonitis." Thesis presented to the University of Nottingham for degree of Doctor of Medicine.

Ashwell, J. D., King, L. B. and Vacchio, M. S. (1996). "Cross-talk between the T cell antigen receptor and the glucocorticoid receptor regulates thymocyte development." Stem Cells **14**(5): 490-500.

Auphan, N., DiDonato, J. A., Rosette, C., Helmberg, A. and Karin, M. (1995). "Immunosupression by glucocorticoids: inhibition of NF-κB activity through induction of IκB synthesis." Science **270**: 286-290.

Axelrod, J. and Reisine, T. D. (1984). "Stress hormones: their interaction and regulation." Science **224**(4648): 452-9.

Baker, A. J., Mooney, A., Hughes, J., Lombardi, D., Johnson, R. J. and Savill, J. (1994). "Mesangial cell apoptosis: the major mechanism for resolution of glomerular hypercellularity in experimental mesangial proliferative nephritis." J Clin Invest **94**(5): 2105-16.

Bamberger, C. M., Bamberger, A. M., de Castro, M. and Chrousos, G. P. (1995). "Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans." J Clin Invest **95**(6): 2435-41.

Barnes, P. J. (1998). "Anti-inflammatory actions of glucocorticoids: molecular mechanisms." Clin Sci (Lond) **94**(6): 557-72.

Beato, M. (1989). "Gene regulation by steroid hormones." Cell **56**: 335-344.

Beato, M., Chavez, S. and Truss, M. (1996). "Transcriptional regulation by steroid hormones." Steroids **61**(4): 240-251.

Beelen, R. H. and Walker, W. S. (1983). "Dynamics of cytochemically distinct subpopulations of macrophages in elicited rat peritoneal exudates." Cell Immunol **82**(2): 246-57.

Bellingan, G. J., Caldwell, H., Howie, S. E., Dransfield, I. and Haslett, C. (1996). "In vivo fate of the inflammatory macrophage during the resolution of inflammation: inflammatory macrophages do not die locally, but emigrate to the draining lymph nodes." J Immunol **157**(6): 2577-85.

Benediktsson, R., Lindsay, R., Noble, J., Seckl, J. R. and Edwards, C. R. W. (1993). "Glucocorticoid exposure in utero: a new model for adult hypertension." Lancet **341**: 339-341.

Berger, J., Tanen, M., Elbrecht, A., Hermanowski-Vosatka, A., Moller, D. E., Wright, S. D. and Thieringer, R. (2001). "Peroxisome proliferator-activated receptor-gamma ligands inhibit adipocyte 11beta -hydroxysteroid dehydrogenase type 1 expression and activity." J Biol Chem **276**(16): 12629-35.

Bertini, R., Bianchi, M. and Ghezzi, P. (1988). "Adrenalectomy sensitizes mice to the lethal effects of interleukin 1 and tumor necrosis factor." J Exp Med **167**(5): 1708-12.

Botto, M., Dell'Agnola, C., Bygrave, A. E., Thompson, E. M., Cook, H. T., Petry, F., Loos, M., Pandolfi, P. P. and Walport, M. J. (1998). "Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies." Nat Genet **19**(1): 56-9.

Bramley, T. (2003). "Non-genomic progesterone receptors in the mammalian ovary; some unresolved issues." Reproduction **125**(1): 3-15.

Brown, R. W., Chapman, K. E., Kotelevtsev, Y., Yau, J. L., Lindsay, R. S., Brett, L., Leckie, C., Murad, P., Lyons, V., Mullins, J. J., Edwards, C. R. and Seckl, J. R. (1996). "Cloning and production of antisera to human placental 11 beta-hydroxysteroid dehydrogenase type 2." Biochem J **313**(Pt 3): 1007-17.

Brown, R. W., Chapman, K. E., Murad, P., Edwards, C. R. and Seckl, J. R. (1996). "Purification of 11 beta-hydroxysteroid dehydrogenase type 2 from human placenta utilizing a novel affinity labelling technique." Biochem J **313**(Pt 3): 997-1005.

Brown, S., Heinisch, I., Ross, E., Shaw, K., Buckley, C. D. and Savill, J. (2002). "Apoptosis disables CD31-mediated cell detachment from phagocytes promoting binding and engulfment." Nature **418**(6894): 200-3.

Brun, R. P., Tontonoz, P., Forman, B. M., Ellis, R., Chen, J., Evans, R. M. and Spiegelman, B. M. (1996). "Differential activation of adipogenesis by multiple PPAR isoforms." Genes & Development **10**: 974-984.

Bujalska, I. J., Kumar, S. and Stewart, P. M. (1997). "Does central obesity reflect "Cushing's disease of the omentum"?" Lancet **349**: 1210-1213.

Cato, A. C. and Wade, E. (1996). "Molecular mechanisms of anti-inflammatory action of glucocorticoids." Bioessays **18**(5): 371-8.

Chikanza, I. C., Petrou, P., Kingsley, G., Chrousos, G. and Panayi, G. S. (1992). "Defective hypothalamic response to immune and inflammatory stimuli in patients with rheumatoid arthritis." Arthritis Rheum **35**(11): 1281-8.

Chinetti, G., Griglio, S., Antonucci, M., Torra, I. P., Delerive, P., Majd, Z., Fruchart, J. C., Chapman, J., Najib, J. and Staels, B. (1998). "Activation of proliferator-activated receptors alpha and gamma induces apoptosis of human monocyte-derived macrophages." J Biol Chem **273**(40): 25573-80.

Chinnaiyan, A. M., O'Rourke, K., Lane, B. R. and Dixit, V. M. (1997). "Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death." Science **275**(5303): 1122-6.

Chomczynski, P. and Sacchi, N. (1987). "Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction." Anal Biochem **162**(1): 156-9.

Cox, G., Crossley, J. and Xing, Z. (1995). "Macrophage engulfment of apoptotic neutrophils contributes to the resolution of acute pulmonary inflammation in vivo." Am J Respir Cell Mol Biol **12**(2): 232-7.

Dallman, M. F., Strack, A. M., Akana, S. F., Bradbury, M. J., Hanson, E. S., Scribner, K. A. and Smith, M. (1993). "Feast and famine: critical role of glucocorticoids with insulin in daily energy flow." Front Neuroendocrinol **14**(4): 303-47.

De Kloet, E. R., Rosenfeld, P., Van Eekelen, J. A. M., Sutano, W. and Levine, S. (1988). Stress, glucocorticoids and development. Progress in Brain Research, Elsevier Science Publishers B. V. **73:** 101-119.

Delerive, P., De Bosscher, K., Besnard, S., Vanden Berghe, W., Peters, J. M., Gonzalez, F. J., Fruchart, J. C., Tedgui, A., Haegeman, G. and Staels, B. (1999). "Peroxisome proliferator-activated receptor alpha negatively regulates the vascular inflammatory gene response by negative cross-talk with transcription factors NF-kappaB and AP-1." J Biol Chem **274**(45): 32048-54.

Di Rosa, M., Radomski, M., Carnuccio, R. and Moncada, S. (1990). "Glucocorticoids inhibit the induction of nitric oxide synthase in macrophages." Biochem Biophys Res Commun **172**(3): 1246-52.

Duffield, J. S., Erwig, L. P., Wei, X., Liew, F. Y., Rees, A. J. and Savill, J. S. (2000). "Activated macrophages direct apoptosis and suppress mitosis of mesangial cells." J Immunol **164**(4): 2110-9.

Edwards, C. R. W., Stewart, P. M., Burt, D., Brett, L., McIntyre, M. A., Sutanto, W. S., de Kloet, E. R. and Monder, C. (1988). "Localisation of 11ß-hydroxysteroid dehydrogenase-tissue specific protector of the mineralocorticoid receptor." Lancet **ii**: 986-989.

Elenkov, I. J. and Chrousos, G. P. (1999). "Stress Hormones, Th1/Th2 patterns, Pro/Anti-inflammatory Cytokines and Susceptibility to Disease." Trends Endocrinol Metab **10**(9): 359-368.

Erwig, L. P., Gordon, S., Walsh, G. M. and Rees, A. J. (1999). "Previous uptake of apoptotic neutrophils or ligation of integrin receptors downmodulates the ability of macrophages to ingest apoptotic neutrophils." Blood **93**(4): 1406-12.

Erwig, L. P., Kluth, D. C., Walsh, G. M. and Rees, A. J. (1998). "Initial cytokine exposure determines function of macrophages and renders them unresponsive to other cytokines." J Immunol **161**(4): 1983-8.

Escher, G., Galli, I., Vishwanath, B. S., Frey, B. M. and Frey, F. J. (1997). "Tumor necrosis factor alpha and interleukin 1beta enhance the cortisone/cortisol shuttle." J Exp Med **186**(2): 189-98.

Ezekowitz, R. A. and Gordon, S. (1982). "Down-regulation of mannosyl receptor-mediated endocytosis and antigen F4/80 in bacillus Calmette-Guerin-activated mouse macrophages. Role of T lymphocytes and lymphokines." J Exp Med **155**(6): 1623-37.

Fadok, V. A., Bratton, D. L., Konowal, A., Freed, P. W., Westcott, J. Y. and Henson, P. M. (1998). "Macrophages that have ingested apoptotic cells in vitro inhibit

proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF." J Clin Invest **101**(4): 890-8.

Fadok, V. A., Bratton, D. L., Rose, D. M., Pearson, A., Ezekewitz, R. A. and Henson, P. M. (2000). "A receptor for phosphatidylserine-specific clearance of apoptotic cells [see comments]." Nature **405**(6782): 85-90.

Fadok, V. A., Savill, J. S., Haslett, C., Bratton, D. L., Doherty, D. E., Campbell, P. A. and Henson, P. M. (1992). "Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells." J Immunol **149**(12): 4029-35.

Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L. and Henson, P. M. (1992). "Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages." J Immunol **148**(7): 2207-16.

Falasca, L., Bergamini, A., Serafino, A., Balabaud, C. and Dini, L. (1996). "Human Kupffer cell recognition and phagocytosis of apoptotic peripheral blood lymphocytes." Exp Cell Res **224**(1): 152-62.

Finney, R. S. H. and Somers, G. F. (1958). "The anti-inflammatory activity of glycyrretinic acid and derivatives." J. Pharm Pharmacol **10**: 613-620.

Finotto, S., Krieglstein, K., Schober, A., Deimling, F., Lindner, K., Bruhl, B., Beier, K., Metz, J., GarciaArraras, J. E., RoigLopez, J. L., Monaghan, P., Schmid, W., Cole, T. J., Kellendonk, C., Tronche, F., Schutz, G. and Unsicker, K. (1999). "Analysis of mice carrying targeted mutations of the glucocorticoid receptor gene argues against an essential role of glucocorticoid signalling for generating adrenal chromaffin cells." Development **126**(13): 2935-2944.

Fryer, C. J. and Archer, T. K. (1998). "Chromatin remodelling by the glucocorticoid receptor requires the BRG1 complex." Nature **393**(6680): 88-91.

Funder, J. W., Pearce, P. T., Smith, R. and Smith, A. I. (1988). "Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated." Science **242**: 583-585.

Galon, J., Franchimont, D., Hiroi, N., Frey, G., Boettner, A., Ehrhart-Bornstein, M., O'Shea, J. J., Chrousos, G. P. and Bornstein, S. R. (2002). "Gene profiling reveals unknown enhancing and suppressive actions of glucocorticoids on immune cells." Faseb J **16**(1): 61-71.

Giles, K. M., Ross, K., Rossi, A. G., Hotchin, N. A., Haslett, C. and Dransfield, I. (2001). "Glucocorticoid augmentation of macrophage capacity for phagocytosis of apoptotic cells is associated with reduced p130Cas expression, loss of paxillin/pyk2 phosphorylation, and high levels of active Rac." J Immunol **167**(2): 976-86.

Gingras, M. C. and Margolin, J. F. (2000). "Differential expression of multiple unexpected genes during U937 cell and macrophage differentiation detected by suppressive subtractive hybridization." Exp Hematol **28**(3): 350.

Goulding, N. J. and Guyre, P. M. (1993). "Glucocorticoids, lipocortins and the immune response." Curr Opin Immunol **5**(1): 108-13.

Greenberg, S. and Grinstein, S. (2002). "Phagocytosis and innate immunity." Curr Opin Immunol **14**(1): 136-45.

Hall, S. E., Savill, J. S., Henson, P. M. and Haslett, C. (1994). "Apoptotic neutrophils are phagocytosed by fibroblasts with participation of the fibroblast vitronectin receptor and involvement of a mannose/fucose-specific lectin." J Immunol **153**(7): 3218-27.

Hamilton, J. A. (1993). "Colony stimulating factors, cytokines and monocyte-macrophages--some controversies." Immunol Today **14**(1): 18-24.

Hammami, M. M. and Siiteri, P. K. (1991). "Regulation of 11ß-hydroxysteroid dehydrogenase activity in human skin fibroblasts: enzymatic modulation of glucocorticoid action." J Clin Endocrinol Metab **73**: 326-334.

Hammond, G. L., Smith, C. L., Paterson, N. A. and Sibbald, W. J. (1990). "A role for corticosteroid-binding globulin in delivery of cortisol to activated neutrophils." J Clin Endocrinol Metab **71**(1): 34-9.

Hannah, S., Nadra, I., Dransfield, I., Pryde, J. G., Rossi, A. G. and Haslett, C. (1998). "Constitutive neutrophil apoptosis in culture is modulated by cell density independently of beta2 integrin-mediated adhesion." FEBS Lett **421**(2): 141-6.

Harper, L., Ren, Y., Savill, J., Adu, D. and Savage, C. O. (2000). "Antineutrophil cytoplasmic antibodies induce reactive oxygen-dependent dysregulation of primed neutrophil apoptosis and clearance by macrophages." Am J Pathol **157**(1): 211-20.

Harris, H. J., Kotelevtsev, Y., Mullins, J. J., Seckl, J. R. and Holmes, M. C. (2001). "Intracellular regeneration of glucocorticoids by 11beta-hydroxysteroid dehydrogenase (11beta-HSD)-1 plays a key role in regulation of the hypothalamic-pituitary-adrenal axis: analysis of 11beta-HSD-1-deficient mice." Endocrinology **142**(1): 114-20.

Hart, S. P., Dougherty, G. J., Haslett, C. and Dransfield, I. (1997). "CD44 regulates phagocytosis of apoptotic neutrophil granulocytes, but not apoptotic lymphocytes, by human macrophages." J Immunol **159**(2): 919-25.

Haslett, C. (1992). "Resolution of acute inflammation and the role of apoptosis in the tissue fate of granulocytes." Clin Sci (Lond) **83**(6): 639-48.

Haslett, C., Lee, A., Savill, J. S., Meagher, L. and Whyte, M. K. (1991). "Apoptosis (programmed cell death) and functional changes in aging neutrophils. Modulation by inflammatory mediators." Chest **99**(3 Suppl): 6S.

Hauner, H., Entenmann, G., Wabitsch, M., Gaillard, D., Ailhaud, G., Negrel, R. and Pfeiffer, E. F. (1989). "Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium." J Clin Invest **84**(5): 1663-70.

Hecht, K., CarlstedtDuke, J., Stierna, P., Gustafsson, J. A., Bronnegard, M. and Wikstrom, A. C. (1997). "Evidence that the beta-isoform of the human glucocorticoid receptor does not act as a physiologically significant repressor." Journal of Biological Chemistry **272**(42): 26659-26664.

Hennebold, J. D., Mu, H. H., Poynter, M. E., Chen, X. P. and Daynes, R. A. (1997). "Active catabolism of glucocorticoids by 11 beta-hydroxysteroid dehydrogenase in vivo is a necessary requirement for natural resistance to infection with Listeria monocytogenes." International Immunology **9**(1): 105-115.

Hennebold, J. D., Ryu, S. Y., Mu, H. H., Galbraith, A. and Daynes, R. A. (1996). "11 beta-hydroxysteroid dehydrogenase modulation of glucocorticoid activities in lymphoid organs." Am J Physiol **270**(6 Pt 2): R1296-306.

Henson, P. M. and Johnston, R. B., Jr. (1987). "Tissue injury in inflammation. Oxidants, proteinases, and cationic proteins." J Clin Invest **79**(3): 669-74.

Hermanowski-Vosatka, A., Gerhold, D., Mundt, S. S., Loving, V. A., Lu, M., Chen, Y., Elbrecht, A., Wu, M., Doebber, T., Kelly, L., Milot, D., Guo, Q., Wang, P. R., Ippolito, M., Chao, Y. S., Wright, S. D. and Thieringer, R. (2000). "PPARalpha agonists reduce 11beta-hydroxysteroid dehydrogenase type 1 in the liver." Biochem Biophys Res Commun **279**(2): 330-6.

Huang, J. T., Welch, J. S., Ricote, M., Binder, C. J., Willson, T. M., Kelly, C., Witztum, J. L., Funk, C. D., Conrad, D. and Glass, C. K. (1999). "Interleukin-4-dependent production of PPAR-gamma ligands in macrophages by 12/15-lipoxygenase." Nature **400**(6742): 378-82.

Hughes, J., Liu, Y., Van Damme, J. and Savill, J. (1997). "Human glomerular mesangial cell phagocytosis of apoptotic neutrophils: mediation by a novel CD36-independent vitronectin receptor/thrombospondin recognition mechanism that is uncoupled from chemokine secretion." J Immunol **158**(9): 4389-97.

Jamieson, C. A. and Yamamoto, K. R. (2000). "Crosstalk pathway for inhibition of glucocorticoid-induced apoptosis by T cell receptor signaling." Proc Natl Acad Sci U S A **97**(13): 7319-24.

Jamieson, P. M., Chapman, K. E., Edwards, C. R. W. and Seckl, J. R. (1995). "11ß-hydroxysteroid dehydrogenase is an exclusive 11ß-reductase in primary cultures of rat hepatocytes: effect of physicochemical and hormonal manipulations." Endocrinology **136**: 4754-4761.

Jamieson, P. M., Walker, B. R., Chapman, K. E., Andrew, R., Rossiter, S. and Seckl, J. R. (2000). "11 beta-hydroxysteroid dehydrogenase type 1 is a predominant 11 beta- reductase in the intact perfused rat liver." J Endocrinol **165**(3): 685-92.

Janeway CA, T. P., Walport M, Capra JD (1999). "Immunobiology." Elsevier Science Ltd/Garland Publishing.

Jiang, C., Ting, A. T. and Seed, B. (1998). "PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines." Nature **391**(6662): 82-6.

Jones, S. T., Denton, J., Holt, P. J. and Freemont, A. J. (1993). "Possible clearance of effete polymorphonuclear leucocytes from synovial fluid by cytophagocytic mononuclear cells: implications for pathogenesis and chronicity in inflammatory arthritis." Ann Rheum Dis **52**(2): 121-6.

Karlsson, A., Follin, P., Leffler, H. and Dahlgren, C. (1998). "Galectin-3 activates the NADPH-oxidase in exudated but not peripheral blood neutrophils." Blood **91**(9): 3430-8.

Kerr, J. F., Wyllie, A. H. and Currie, A. R. (1972). "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics." Br J Cancer **26**(4): 239-57.

Kitamura, M., Burton, S., English, J., Kawachi, H. and Fine, L. G. (1995). "Transfer of a mutated gene encoding active transforming growth factor- beta 1 suppresses mitogenesis and IL-1 response in the glomerulus." Kidney Int **48**(6): 1747-57.

Kitraki, E., Karandrea, D. and Kittas, C. (1999). "Long-lasting effects of stress on glucocorticoid receptor gene expression in the rat brain." Neuroendocrinology **69**(5): 331-338.

Kotelevtsev, Y., Holmes, M. C., Burchell, A., Houston, P. M., Schmoll, D., Jamieson, P., Best, R., Brown, R., Edwards, C. R. W., Seckl, J. R. and Mullins, J. J. (1997). "11ß-hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid inducible responses and resist hyperglycaemia on obesity or stress." Proc Natl Acad Sci USA **94**: 14924-14929.

Kralli, A., Bohen, S. P. and Yamamoto, K. R. (1995). "Lem1, an Atp-Binding-Cassette Transporter, Selectively Modulates the Biological Potency of Steroid-Hormones." Proceedings of the National Academy of Sciences of the United States of America **92**(10): 4701-4705.

Krozowski, Z., Albiston, A. L., Obeyesekere, V. R., Andrews, R. K. and Smith, R. E. (1995). "The human 11 beta-hydroxysteroid dehydrogenase type II enzyme: comparisons with other species and localization to the distal nephron." J Steroid Biochem Mol Biol **55**(5-6): 457-64.

Krozowski, Z., MaGuire, J. A., Stein-Oakley, A. N., Dowling, J., Smith, R. E. and Andrews, R. K. (1995). "Immunohistochemical localization of the 11 beta-hydroxysteroid dehydrogenase type II enzyme in human kidney and placenta." J Clin Endocrinol Metab **80**(7): 2203-9.

Lagasse, E. and Weissman, I. L. (1996). "Flow cytometric identification of murine neutrophils and monocytes." J Immunol Methods **197**(1-2): 139-50.

Lakshmi, V. and Monder, C. (1985). "Evidence For Independent 11-Oxidase and 11-Reductase Activities of 11-Beta-Hydroxysteroid Dehydrogenase - Enzyme Latency, Phase- Transitions, and Lipid Requirements." Endocrinology **116**(2): 552-560.

Lakshmi, V. and Monder, C. (1985). "Extraction of 11-Beta-Hydroxysteroid Dehydrogenase From Rat-Liver Microsomes By Detergents." Journal of Steroid Biochemistry and Molecular Biology **22**(3): 331-340.

Lakshmi, V. and Monder, C. (1988). "Purification and characterisation of the corticosteroid 11ß-dehydrogenase component of the rat liver 11ß-hydroxysteroid dehydrogenase complex." Endocrinology **123**: 2390-2398.

Lane, S. J., Wilkinson, J. R., Cochrane, G. M., Lee, T. H. and Arm, J. P. (1993). "Differential in vitro regulation by glucocorticoids of monocyte-derived cytokine generation in glucocorticoid-resistant bronchial asthma." Am Rev Respir Dis **147**(3): 690-6.

Lee, A., Whyte, M. K. and Haslett, C. (1993). "Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators." J Leukoc Biol **54**(4): 283-8.

Li, A. C., Guidez, F. R., Collier, J. G. and Glass, C. K. (1998). "The macrosialin promoter directs high levels of transcriptional activity in macrophages dependent on combinatorial interactions between PU.1 and c-Jun." J Biol Chem **273**(9): 5389-99.

Lindsay, R. S., Lindsay, R. M., Edwards, C. R. and Seckl, J. R. (1996). "Inhibition of 11-beta-hydroxysteroid dehydrogenase in pregnant rats and the programming of blood pressure in the offspring." Hypertension **27**(6): 1200-4.

Liu, Y., Cousin, J. M., Hughes, J., Van Damme, J., Seckl, J. R., Haslett, C., Dransfield, I., Savill, J. and Rossi, A. G. (1999). "Glucocorticoids promote nonphlogistic phagocytosis of apoptotic leukocytes." J Immunol **162**(6): 3639-46.

Losel, R. and Wehling, M. (2003). "Nongenomic actions of steroids hormones." Nat Rev Mol Biol. **4**(1): 46-56.

Low, S. C., Assaad, S. N., Rajan, V., Chapman, K. E., Edwards, C. R. and Seckl, J. R. (1993). "Regulation of 11 beta-hydroxysteroid dehydrogenase by sex steroids in

vivo: further evidence for the existence of a second dehydrogenase in rat kidney." J Endocrinol **139**(1): 27-35.

Low, S. C., Chapman, K. E., Edwards, C. R., Wells, T., Robinson, I. C. and Seckl, J. R. (1994). "Sexual dimorphism of hepatic 11 beta-hydroxysteroid dehydrogenase in the rat: the role of growth hormone patterns." J Endocrinol **143**(3): 541-8.

Low, S. C., Chapman, K. E., Edwards, C. R. W. and Seckl, J. R. (1994). "'Liver-type' 11ß-hydroxysteroid dehydrogenase cDNA encodes reductase not dehydrogenase activity in intact mammalian COS-7 cells." J Mol Endocrinol **13**: 167-174.

Low, S. C., Moisan, M.-P., Noble, J. M., Edwards, C. R. W. and Seckl, J. R. (1994). "Glucocorticoids regulate hippocampal 11ß-hydroxysteroid dehydrogenase activity and gene expression in vivo in the rat." J. Neuroendocrinol. **6**: 285-290.

Makino, S., Smith, M. A. and Gold, P. W. (1995). "Increased Expression of Corticotropin-Releasing Hormone and Vasopressin Messenger-Ribonucleic-Acid (Messenger-Rna) in the Hypothalamic Paraventricular Nucleus During Repeated Stress - Association With Reduction in Glucocorticoid Receptor Messenger-Rna Levels." Endocrinology **136**(8): 3299-3309.

Malech, H. L. and Gallin, J. I. (1987). "Current concepts: immunology. Neutrophils in human diseases." N Engl J Med **317**(11): 687-94.

Mandrup-Poulsen, T., Nerup, J., Reimers, J. I., Pociot, F., Andersen, H. U., Karlsen, A., Bjerre, U. and Bergholdt, R. (1995). "Cytokines and the endocrine system. I. The immunoendocrine network." Eur J Endocrinol **133**(6): 660-71.

Masuzaki, H., Paterson, J., Shinyama, H., Morton, N. M., Mullins, J. J., Seckl, J. R. and Flier, J. S. (2001). "A transgenic model of visceral obesity and the metabolic syndrome." Science **294**(5549): 2166-70.

Matsusaka, T., Fujikawa, K., Nishio, Y., Mukaida, N., Matsushima, K., Kishimoto, T. and Akira, S. (1993). "Transcription factors nf-il6 and nf-kappa-b synergistically activate transcription of the inflammatory cytokines, interleukin-6 and

interleukin-8." Proceedings Of the National Academy Of Sciences Of the United States Of America **90**: 10193-10197.

Meagher, L. C., Cousin, J. M., Seckl, J. R. and Haslett, C. (1996). "Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes." J Immunol **156**(11): 4422-8.

Meagher, L. C., Savill, J. S., Baker, A., Fuller, R. W. and Haslett, C. (1992). "Phagocytosis of apoptotic neutrophils does not induce macrophage release of thromboxane B2." J Leukoc Biol **52**(3): 269-73.

Melnicoff, M. J., Horan, P. K., Breslin, E. W. and Morahan, P. S. (1988). "Maintenance of peritoneal macrophages in the steady state." J Leukoc Biol **44**(5): 367-75.

Melnicoff, M. J., Horan, P. K. and Morahan, P. S. (1989). "Kinetics of changes in peritoneal cell populations following acute inflammation." Cell Immunol **118**(1): 178-91.

Metchnikoff, E. (1891). "Lecture VII. In Lectures on the comparative pathology of inflammation. Translated from the French by F. A Starling and E. H Starling." Kegan, Paul, Trench and Trubner, London. **1893**: 107.

Metchnikoff, E. (1891). "Lecture XII. In Lectures on the comparative pathology of inflammation. Translated from French by F. A Starling and E. H Starling." Kegan, Paul, Trench and Trubner, London. **1893**: 107.

Miller, A. H., Spencer, R. L., Pearce, B. D., Pisell, T. L., Azrieli, Y., Tanapat, P., Moday, H., Rhee, R. and McEwen, B. S. (1998). "Glucocorticoid receptors are differentially expressed in the cells and tissues of the immune system." Cellular Immunology **186**(1): 45-54.

Mills, C. D., Kincaid, K., Alt, J. M., Heilman, M. J. and Hill, A. M. (2000). "M-1/M-2 macrophages and the Th1/Th2 paradigm." J Immunol **164**(12): 6166-73.

Monder, C., Stewart, P. M., Lakshmi, V., Valentino, R., Burt, D. and Edwards, C. R. (1989). "Licorice inhibits corticosteroid 11 beta-dehydrogenase of rat kidney and liver: in vivo and in vitro studies." Endocrinology **125**(2): 1046-53.

Morgan, B. P. and Walport, M. J. (1991). "Complement deficiency and disease." Immunol Today **12**(9): 301-6.

Morton, N. M., Holmes, M. C., Fievet, C., Staels, B., Tailleux, A., Mullins, J. J. and Seckl, J. R. (2001). "Improved lipid and lipoprotein profile, hepatic insulin sensitivity, and glucose tolerance in 11beta-hydroxysteroid dehydrogenase type 1 null mice." J Biol Chem **276**(44): 41293-300.

Munck, A., Guyre, P. M. and Holbrook, N. J. (1984). "Physiological functions of glucocorticoids in stress and their relation to pharmacological actions." Endocr Rev **5**(1): 25-44.

Musson, R. A. and Henson, P. M. (1979). "Humoral and formed elements of blood modulate the response of peripheral blood monocytes. I. Plasma and serum inhibit and platelets enhance monocyte adherence." J Immunol **122**(5): 2026-31.

Nakano, T., Ohara, O., Teraoka, H. and Arita, H. (1990). "Glucocorticoids suppress group II phospholipase A2 production by blocking mRNA synthesis and post-transcriptional expression." J Biol Chem **265**(21): 12745-8.

Newman, S. L., Henson, J. E. and Henson, P. M. (1982). "Phagocytosis of senescent neutrophils by human monocyte-derived macrophages and rabbit inflammatory macrophages." J Exp Med **156**(2): 430-42.

Newman, S. L., Musson, R. A. and Henson, P. M. (1980). "Development of functional complement receptors during in vitro maturation of human monocytes into macrophages." J Immunol **125**(5): 2236-44.

Nicholson, D. W. and Thornberry, N. A. (1997). "Caspases: killer proteases." Trends Biochem Sci **22**(8): 299-306.

Ogawa, M. (1993). "Differentiation and proliferation of hematopoietic stem cells." Blood **81**(11): 2844-53.

Otto, C., Reichardt, H. M. and Schutz, G. (1997). "Absence of glucocorticoid receptor-beta in mice." Journal of Biological Chemistry **272**(42): 26665-26668.

Ozols, J. (1995). "Lumenal orientation and posttranslational modifications of the liver microsomal 11ß-hydroxysteroid dehydrogenase." J Biol Chem **270**: 2305-2312.

Pardridge, W. M. (1987). "Plasma protein-mediated transport of steroid and thyroid hormones." Am J Physiol **252**(2 Pt 1): E157-64.

Pepin, M.-C., Pothier, F. and Barden, N. (1992). "Impaired type II glucocorticoid receptor function in mice bearing antisense RNA transgene." Nature **355**: 725-728.

Pfeilschifter, J., Schalkwijk, C., Briner, V. A. and van den Bosch, H. (1993). "Cytokine-stimulated secretion of group II phospholipase A2 by rat mesangial cells. Its contribution to arachidonic acid release and prostaglandin synthesis by cultured rat glomerular cells." J Clin Invest **92**(5): 2516-23.

Poli, V. (1998). "The role of C/EBP isoforms in the control of inflammatory and native immunity functions." J Biol Chem **273**(45): 29279-29282.

Pompei, R., Flore, O., Marccialis, M. A., Pani, A. and Loddo, B. (1979). "Glycyrrhizic acid inhibits virus growth and inactivates virus particles." Nature **281**(5733): 689-90.

Pope, R. M., Leutz, A. and Ness, S. A. (1994). "C/EBP beta regulation of the tumor necrosis factor alpha gene." J Clin Invest **94**(4): 1449-55.

Poznansky, M. C., Gordon, A. C., Douglas, J. G., Krajewski, A. S., Wyllie, A. H. and Grant, I. W. (1984). "Resistance to methylprednisolone in cultures of blood mononuclear cells from glucocorticoid-resistant asthmatic patients." Clin Sci (Lond) **67**(6): 639-45.

Pratt, W. B. (1993). "The Role of Heat-Shock Proteins in Regulating the Function, Folding, and Trafficking of the Glucocorticoid Receptor." Journal of Biological Chemistry **268**(29): 21455-21458.

Purton, J. F., Boyd, R. L., Cole, T. J. and Godfrey, D. I. (2000). "Intrathymic T cell development and selection proceeds normally in the absence of glucocorticoid receptor signaling." Immunity **13**(2): 179-86.

Rajan, V., Chapman, K. E., Lyons, V., Jamieson, P., Mullins, J. J., Edwards, C. R. and Seckl, J. R. (1995). "Cloning, sequencing and tissue-distribution of mouse 11 beta- hydroxysteroid dehydrogenase-1 cDNA." J Steroid Biochem Mol Biol **52**(2): 141-7.

Ray, A. and Prefontaine, K. E. (1994). "Physical association and functional antagonism between the p65 subunit of transcription factor NFκB and the glucocorticoid receptor." Proc Natl Acad Sci USA **91**: 752-756.

Reichardt, H. M., Kaestner, K. H., Tuckermann, J., Kretz, O., Wessely, O., Bock, R., Gass, P., Schmid, W., Herrlich, P., Angel, P. and Schutz, G. (1998). "DNA binding of the glucocorticoid receptor is not essential for survival [see comments]." Cell **93**(4): 531-41.

Ren, Y. and Savill, J. (1995). "Proinflammatory cytokines potentiate thrombospondin-mediated phagocytosis of neutrophils undergoing apoptosis." J Immunol **154**(5): 2366-74.

Riches, D. W. (1995). "Signalling heterogeneity as a contributing factor in macrophage functional diversity." Semin Cell Biol **6**(6): 377-84.

Ricote, M., Li, A. C., Willson, T. M., Kelly, C. J. and Glass, C. K. (1998). "The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation." Nature **391**(6662): 79-82.

Ringold, G. M., Chapman, A. B., Knight, D. M. and Torti, F. M. (1986). "Hormonal control of adipogenesis." Ann N Y Acad Sci **478**: 109-19.

Rosen, E. D., Sarraf, P., Troy, A. E., Bradwin, G., Moore, K., Milstone, D. S., Spiegelman, B. M. and Mortensen, R. M. (1999). "PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro." Mol Cell **4**(4): 611-7.

Sanui, H., Yoshida, S., Nomoto, K., Ohhara, R. and Adachi, Y. (1982). "Peritoneal macrophages which phagocytose autologous polymorphonuclear leucocytes in guinea-pigs. I: induction by irritants and microorgansisms and inhibition by colchicine." Br J Exp Pathol **63**(3): 278-84.

Sapolsky, R. M., Krey, L. C. and McEwen, B. S. (1984). "Stress down-regulates corticosterone receptors in a site-specific manner in the brain." Endocrinology **114**: 287-292.

Savill, J. (1997). "Apoptosis in resolution of inflammation." J Leukoc Biol **61**(4): 375-80.

Savill, J. (1997). "Recognition and phagocytosis of cells undergoing apoptosis." Br Med Bull **53**(3): 491-508.

Savill, J. and Fadok, V. (2000). "Corpse clearance defines the meaning of cell death." Nature **407**(6805): 784-8.

Savill, J., Hogg, N., Ren, Y. and Haslett, C. (1992). "Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis." J Clin Invest **90**(4): 1513-22.

Savill, J., Smith, J., Sarraf, C., Ren, Y., Abbott, F. and Rees, A. (1992). "Glomerular mesangial cells and inflammatory macrophages ingest neutrophils undergoing apoptosis." Kidney Int **42**(4): 924-36.

Savill, J. S., Henson, P. M. and Haslett, C. (1989). "Phagocytosis of aged human neutrophils by macrophages is mediated by a novel "charge-sensitive" recognition mechanism." J Clin Invest **84**(5): 1518-27.

Savill, J. S., Wyllie, A. H., Henson, J. E., Walport, M. J., Henson, P. M. and Haslett, C. (1989). "Macrophage phagocytosis of aging neutrophils in inflammation.

Programmed cell death in the neutrophil leads to its recognition by macrophages." J Clin Invest **83**(3): 865-75.

Sawyer, R. T., Strausbauch, P. H. and Volkman, A. (1982). "Resident macrophage proliferation in mice depleted of blood monocytes by strontium-89." Lab Invest **46**(2): 165-70.

Schinkel, A. H., Smit, J. J. M., Vantellingen, O., Beijnen, J. H., Wagenaar, E., Vandeemter, L., Mol, C., Vandervalk, M. A., Robanusmaandag, E. C., Teriele, H. P. J., Berns, A. J. M. and Borst, P. (1994). "Disruption of the Mouse Mdr1a P-Glycoprotein Gene Leads to a Deficiency in the Blood-Brain-Barrier and to Increased Sensitivity to Drugs." Cell **77**(4): 491-502.

Schleimer, R. P. (1993). "An overview of glucocorticoid anti-inflammatory actions." Eur J Clin Pharmacol **45**(Suppl 1): S3-7; discussion S43-4.

Seckl, J. R., Benediktsson, R., Lindsay, R. S. and Brown, R. W. (1995). "Placental 11ß-hydroxysteroid dehydrogenase and the programming of hypertension." J. Steroid Biochem Molec Biol **55**: 447-455.

Seckl, J. R. and Chapman, K. E. (1997). "Medical and physiological aspects of the 11beta-hydroxysteroid dehydrogenase system." Eur J Biochem **249**(2): 361-4.

Seckl, J. R. and Walker, B. R. (2001). "Minireview: 11beta-hydroxysteroid dehydrogenase type 1- a tissue- specific amplifier of glucocorticoid action." Endocrinology **142**(4): 1371-6.

Shapiro, S. D., Griffin, G. L., Gilbert, D. J., Jenkins, N. A., Copeland, N. G., Welgus, H. G., Senior, R. M. and Ley, T. J. (1992). "Molecular cloning, chromosomal localization, and bacterial expression of a murine macrophage metalloelastase." J Biol Chem **267**(7): 4664-71.

Shimizu, A., Kitamura, H., Masuda, Y., Ishizaki, M., Sugisaki, Y. and Yamanaka, N. (1995). "Apoptosis in the repair process of experimental proliferative glomerulonephritis." Kidney Int **47**(1): 114-21.

Shimizu, A., Masuda, Y., Kitamura, H., Ishizaki, M., Sugisaki, Y. and Yamanaka, N. (1996). "Apoptosis in progressive crescentic glomerulonephritis." Lab Invest **74**(5): 941-51.

Spector, M. S., Desnoyers, S., Hoeppner, D. J. and Hengartner, M. O. (1997). "Interaction between the C. elegans cell-death regulators CED-9 and CED- 4." Nature **385**(6617): 653-6.

Stern, M., Savill, J. and Haslett, C. (1996). "Human monocyte-derived macrophage phagocytosis of senescent eosinophils undergoing apoptosis. Mediation by alpha v beta 3/CD36/thrombospondin recognition mechanism and lack of phlogistic response." Am J Pathol **149**(3): 911-21.

Sternberg, E. M. (2001). "Neuroendocrine regulation of autoimmune/inflammatory disease." J Endocrinol **169**(3): 429-35.

Stewart, P. M. and Krozowski, Z. S. (1999). "11 beta-Hydroxysteroid dehydrogenase." Vitam Horm **57**: 249-324.

Stewart, P. M., Valentino, R., Wallace, A. M., Burt, D., Shackleton, C. H. L. and Edwards, C. R. W. (1987). "Mineralocorticoid activity of liquorice: 11ß-hydroxysteroid dehydrogenase deficiency comes of age." Lancet **ii**: 821-824.

Stewart, P. M., Wallace, A. M., Atherden, S. M., Shearing, C. H. and Edwards, C. R. (1990). "Mineralocorticoid activity of carbenoxolone: contrasting effects of carbenoxolone and liquorice on 11 beta-hydroxysteroid dehydrogenase activity in man." Clin Sci (Lond) **78**(1): 49-54.

Stuart, L. M., Lucas, M., Simpson, C., Lamb, J., Savill, J. and Lacy-Hulbert, A. (2002). "Inhibitory effects of apoptotic cell ingestion upon endotoxin-driven myeloid dendritic cell maturation." J Immunol **168**(4): 1627-35.

Sugiyama, H., Kashihara, N., Makino, H., Yamasaki, Y. and Ota, A. (1996). "Apoptosis in glomerular sclerosis." Kidney Int **49**(1): 103-11.

Sugiyama, H., Savill, J. S., Kitamura, M., Zhao, L. and Stylianou, E. (1999). "Selective sensitization to tumor necrosis factor-alpha-induced apoptosis by blockade of NF-kappaB in primary glomerular mesangial cells." J Biol Chem **274**(28): 19532-7.

Takeda, Y., Watanabe, H., Yonehara, S., Yamashita, T., Saito, S. and Sendo, F. (1993). "Rapid acceleration of neutrophil apoptosis by tumor necrosis factor- alpha." Int Immunol **5**(6): 691-4.

Tanaka, T., Akira, S., Yoshida, K., Umemoto, M., Yoneda, Y., Shirafuji, N., Fujiwara, H., Suematsu, S., Yoshida, N. and Kishimoto, T. (1995). "Targeted disruption of the nf-il6 gene discloses its essential role in bacteria killing and tumor-cytotoxicity by macrophages." Cell **80**: 353-361.

Tavor, S., Vuong, P. T., Park, D. J., Gombart, A. F., Cohen, A. H. and Koeffler, H. P. (2002). "Macrophage functional maturation and cytokine production are impaired in C/EBP epsilon-deficient mice." Blood **99**(5): 1794-801.

Taylor, P. R., Carugati, A., Fadok, V. A., Cook, H. T., Andrews, M., Carroll, M. C., Savill, J. S., Henson, P. M., Botto, M. and Walport, M. J. (2000). "A hierarchical role for classical pathway complement proteins in the clearance of apoptotic cells in vivo." J Exp Med **192**(3): 359-66.

Teelucksingh, S., Mackie, A. D. R., Burt, D., McIntyre, M. A., Brett, L. and Edwards, C. R. W. (1990). "Potentiation of Hydrocortisone Activity in Skin By Glycyrrhetinic Acid." Lancet **335**(8697): 1060-1063.

Tetsuka, M., Haines, L. C., Milne, M., Simpson, G. E. and Hillier, S. G. (1999). "Regulation of 11beta-hydroxysteroid dehydrogenase type 1 gene expression by LH and interleukin-1beta in cultured rat granulosa cells." J Endocrinol **163**(3): 417-23.

Tetsuka, M., Milne, M., Simpson, G. E. and Hillier, S. G. (1999). "Expression of 11ß-hydroxysteroid dehydrogenase, glucocorticoid receptor, and mineralocorticoid receptor genes in rat ovary." Biol Reprod **60**(2): 330-335.

Tetsuka, M., Thomas, F. J., Thomas, M. J., Anderson, R. A., Mason, J. I. and Hillier, S. G. (1997). "Differential expression of messenger ribonucleic acids encoding 11ß-hydroxysteroid dehydrogenase types 1 and 2 in human granulosa cells." J Clin Endocrinol Metab **82**: 2006-2009.

Thieringer, R., Le Grand, C. B., Carbin, L., Cai, T. Q., Wong, B., Wright, S. D. and Hermanowski-Vosatka, A. (2001). "11 Beta-hydroxysteroid dehydrogenase type 1 is induced in human monocytes upon differentiation to macrophages." J Immunol **167**(1): 30-5.

Tomlinson, J. W., Draper, N., Mackie, J., Johnson, A. P., Holder, G., Wood, P. and Stewart, P. M. (2002). "Absence of Cushingoid phenotype in a patient with Cushing's disease due to defective cortisone to cortisol conversion." J Clin Endocrinol Metab **87**(1): 57-62.

Tomlinson, J. W., Moore, J., Cooper, M. S., Bujalska, I., Shahmanesh, M., Burt, C., Strain, A., Hewison, M. and Stewart, P. M. (2001). "Regulation of expression of 11beta-hydroxysteroid dehydrogenase type 1 in adipose tissue: tissue-specific induction by cytokines." Endocrinology **142**(5): 1982-9.

Tontonoz, P., Nagy, L., Alvarez, J. G., Thomazy, V. A. and Evans, R. M. (1998). "PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL." Cell **93**(2): 241-52.

Trinchieri, G. (1997). "Cytokines acting on or secreted by macrophages during intracellular infection (IL-10, IL-12, IFN-gamma)." Curr Opin Immunol **9**(1): 17-23.

Truss, M., Bartsch, J., Schelbert, A., Hache, R. J. G. and Beato, M. (1995). "Hormone Induces Binding of Receptors and Transcription Factors to a Rearranged Nucleosome On the Mmtv Promoter in-Vivo." Embo Journal **14**(8): 1737-1751.

Vacchio, M. S. and Ashwell, J. D. (1997). "Thymus-derived glucocorticoids regulate antigen-specific positive selection." Journal of Experimental Medicine **185**(11): 2033-2038.

Vacchio, M. S., Papadopoulos, V. and Ashwell, J. D. (1994). "Steroid-Production in the Thymus - Implications For Thymocyte Selection." Journal of Experimental Medicine **179**(6): 1835-1846.

Valledor, A. F., Borras, F. E., Cullell-Young, M. and Celada, A. (1998). "Transcription factors that regulate monocyte/macrophage differentiation." J Leukoc Biol **63**(4): 405-17.

Van Furth, R., Diesselhoff-den Dulk, M. C. and Mattie, H. (1973). "Quantitative study on the production and kinetics of mononuclear phagocytes during an acute inflammatory reaction." J Exp Med **138**(6): 1314-30.

Vanderbilt, J. N., Miesfeld, R., Maler, B. A. and Yamamoto, K. R. (1987). "Intracellular receptor concentration limits glucocorticoid-dependent enhancer activity." Mol Endocrinol **1**(1): 68-74.

Vaux, D. L. and Korsmeyer, S. J. (1999). "Cell death in development." Cell **96**(2): 245-54.

Vazquez-Torres, A. and Fang, F. C. (2001). "Oxygen-dependent anti-Salmonella activity of macrophages." Trends Microbiol **9**(1): 29-33.

Vishwanath, B. S., Frey, F. J., Bradbury, M. J., Dallman, M. F. and Frey, B. M. (1993). "Glucocorticoid deficiency increases phospholipase A2 activity in rats." J Clin Invest **92**(4): 1974-80.

Voll, R. E., Herrmann, M., Roth, E. A., Stach, C., Kalden, J. R. and Girkontaite, I. (1997). "Immunosuppressive effects of apoptotic cells [letter]." Nature **390**(6658): 350-1.

Whitworth, J. A., Stewart, P. M., Burt, D., Atherden, S. M. and Edwards, C. R. (1989). "The kidney is the major site of cortisone production in man." Clin Endocrinol (Oxf) **31**(3): 355-61.

Whorwood, C., Sheppard, M. and Stewart, P. (1993). "Tissue specific effects of thyroid hormone on 11ß-hydroxysteroid dehydrogenase gene expression." Journal of Steroid Biochemistry and Molecular Biology **46**(5): 539-547.

Whyte, M. K., Meagher, L. C., MacDermot, J. and Haslett, C. (1993). "Impairment of function in aging neutrophils is associated with apoptosis." J Immunol **150**(11): 5124-34.

Williams, L. J., Lyons, V., MacLeod, I., Rajan, V., Darlington, G. J., Poli, V., Seckl, J. R. and Chapman, K. E. (2000). "C/EBP regulates hepatic transcription of 11beta -hydroxysteroid dehydrogenase type 1. A novel mechanism for cross-talk between the C/EBP and glucocorticoid signaling pathways." J Biol Chem **275**(39): 30232-9.

Wu, Z., Rosen, E. D., Brun, R., Hauser, S., Adelmant, G., Troy, A. E., McKeon, C., Darlington, G. J. and Spiegelman, B. M. (1999). "Cross-regulation of C/EBP alpha and PPAR gamma controls the transcriptional pathway of adipogenesis and insulin sensitivity." Mol Cell **3**(2): 151-8.

Wyllie, A. H., Kerr, J. F. and Currie, A. R. (1980). "Cell death: the significance of apoptosis." Int Rev Cytol **68**: 251-306.

Yau, J. L., Noble, J., Kenyon, C. J., Hibberd, C., Kotelevtsev, Y., Mullins, J. J. and Seckl, J. R. (2001). "Lack of tissue glucocorticoid reactivation in 11beta -hydroxysteroid dehydrogenase type 1 knockout mice ameliorates age-related learning impairments." Proc Natl Acad Sci U S A **98**(8): 4716-21.

Appendix 1:

Time-course of sterile peritonitis

Both WT and 11β-HSD1-deficient mice were injected i.p with 1ml 3% thioglycollate, and peritoneal cells were harvested over the course of 4d. F4/80 and GR-1 positive cells of the peritoneal lavages were assessed by flow cytometry. Cytospins of these cells were also prepared for morphological examination.

Control mice:

Figure 7-1A shows that, in a non-inflammatory state the resident peritoneal cells separate into 3 distinct populations based on forward and side scatter properties. There is no difference in the F4/80 properties of the RP Mϕs in either the WT or 11β-HSD1-deficient mice, with the majority of the F4/80 positive cells lying in gate R7 (Fig.7-1B). There are minimal numbers of PMNs present in the peritoneum of these control mice and any GR-1 positivity detected in lavages of both genotypes is representative of contamination by peripheral blood during surgery (Fig.7-1B). These observations are consistent with analysis of the eosin and haematoxylin stained cytospins shown in Figure 7-2.

4h after onset of TE peritonitis:

Cells harvested at 4h do not retain the strong F4/80 positivity seen before peritonitis is induced. Staining in the R7 population is weak and detectable in the 2nd log order in both R8 and R9 populations (Fig.7-3B). There is a significant increase in the number of cells in the R9 population (Fig.7-3A). Figure 7-3B shows that these cells are positive for GR-1 and are confirmed as infiltrating PMNs by morphological analysis (Fig.7-4). There is debate as to whether RP Mϕs lose surface F4/80 expression, emigrate or adhere to the peritoneal wall. However, cytospins indicate that they are still present in the peritoneum in significant numbers so it is difficult to explain their weak staining (Fig.7-4). No differences were detected between WT and 11β-HSD1-deficient cell populations.

1d after onset of TE peritonitis:

There is strong F4/80 staining in all 3 populations of cells harvested 1d after the onset of peritonitis (Fig.7-5A). In contrast, low staining for GR-1 is observed in R7 and R8, but staining is strong in R9 (Fig.7-5B). However, GR-1 staining is lower than seen at 4h,

and this is representative of fewer PMNs present in the lavages at this later time-point (Fig.7-6). It is of interest that low numbers of free aPMNs and evidence of ingestion within Mϕs are detectable in the WT cells (Fig.7-6A). Such occurance was not apparent in the 11β-HSD1-deficient cells. However no gross differences in F4/80 and GR-1 staining between the genotypes were observed.

2d after onset of TE peritonitis:

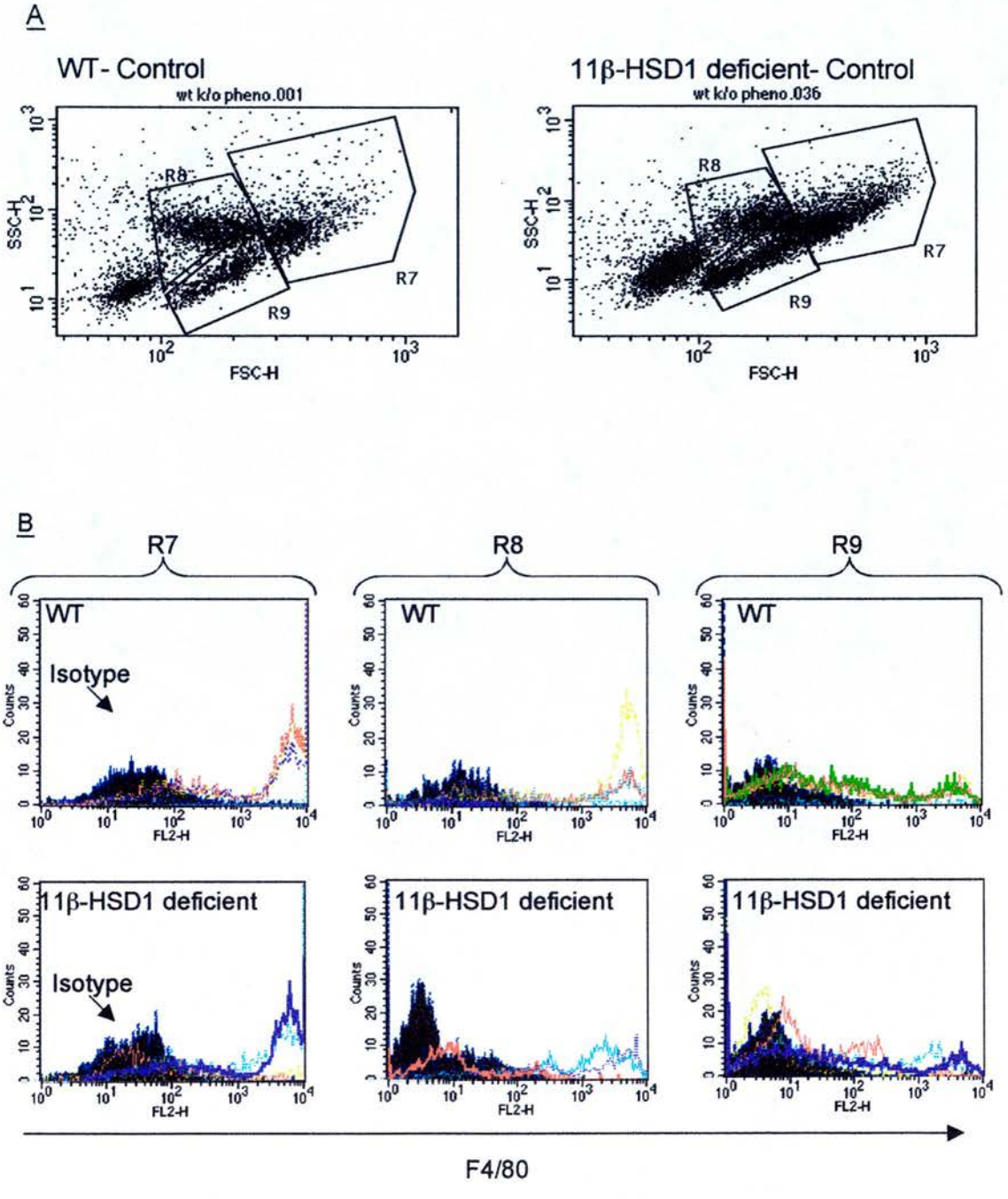
At d2, there is strong F4/80 staining in all 3 populations, whereas GR-1 staining is lower than d1 (Fig.7-7). Again no differences are visible between the 2 genotypes by flow cytometry, however there are clearly greater numbers of free aPMNs and TEP Mϕs containing recently ingested aPMNs in the lavages from 11β-HSD1-deficient mice (Fig.7-8B).

3d after onset of peritonitis:

There is still a strong F4/80 staining in both WT and 11β-HSD1 cells, however GR-1 staining is minimal (Fig.7-9). This is consistent with the cytospins, which show predominantly TEP Mϕs and very few PMNs (Fig.7-10). A greater number of phagocytic 'events' are observed in 11β-HSD1-deficient cells than WT, although very few free aPMNs are now observed (Fig. 7-10B).

4d after onset of peritonitis:

The peritonitis has fully resolved. flow cytometry detects no GR-1 positivity and no PMNs are apparent in the cytospins (Figs. 7-11B and 7-12B). All 3 populations stain strongly for F4/80 and R8 now shows a bi-modal distribution (Fig.7-11A). No differences are observed between the genotypes.


A
WT- Control
wt k/o pheno.001
11β-HSD1 deficient- Control
wt k/o pheno.036
R8
R7
R9
SSC-H
FSC-H
B
R7
R8
R9
WT
Isotype
11β-HSD1 deficient
Counts
FL2-H
F4/80

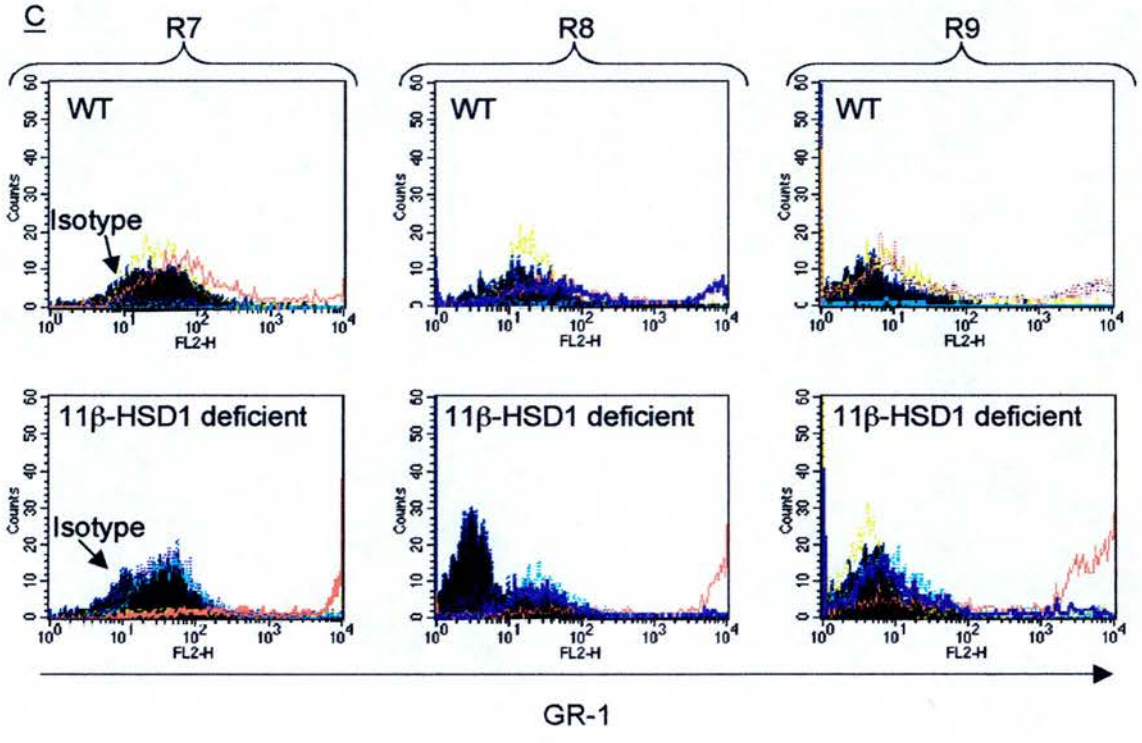
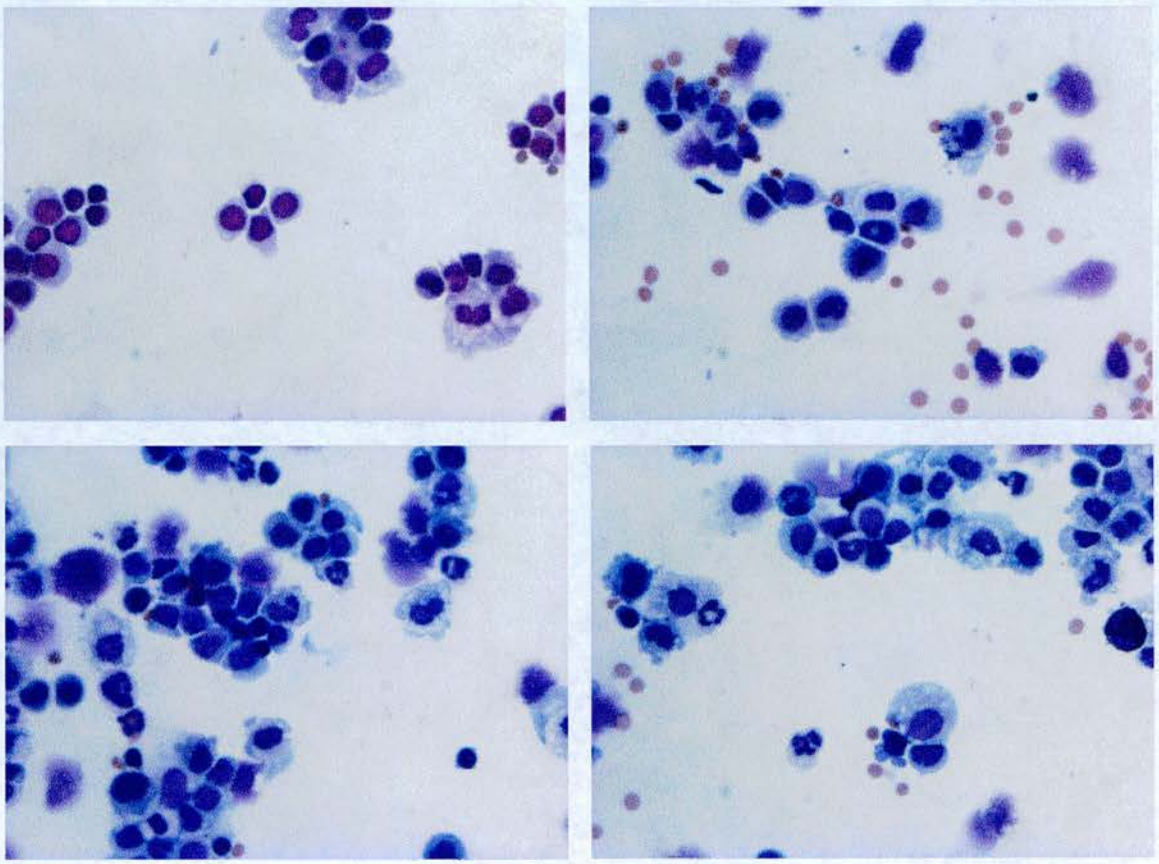
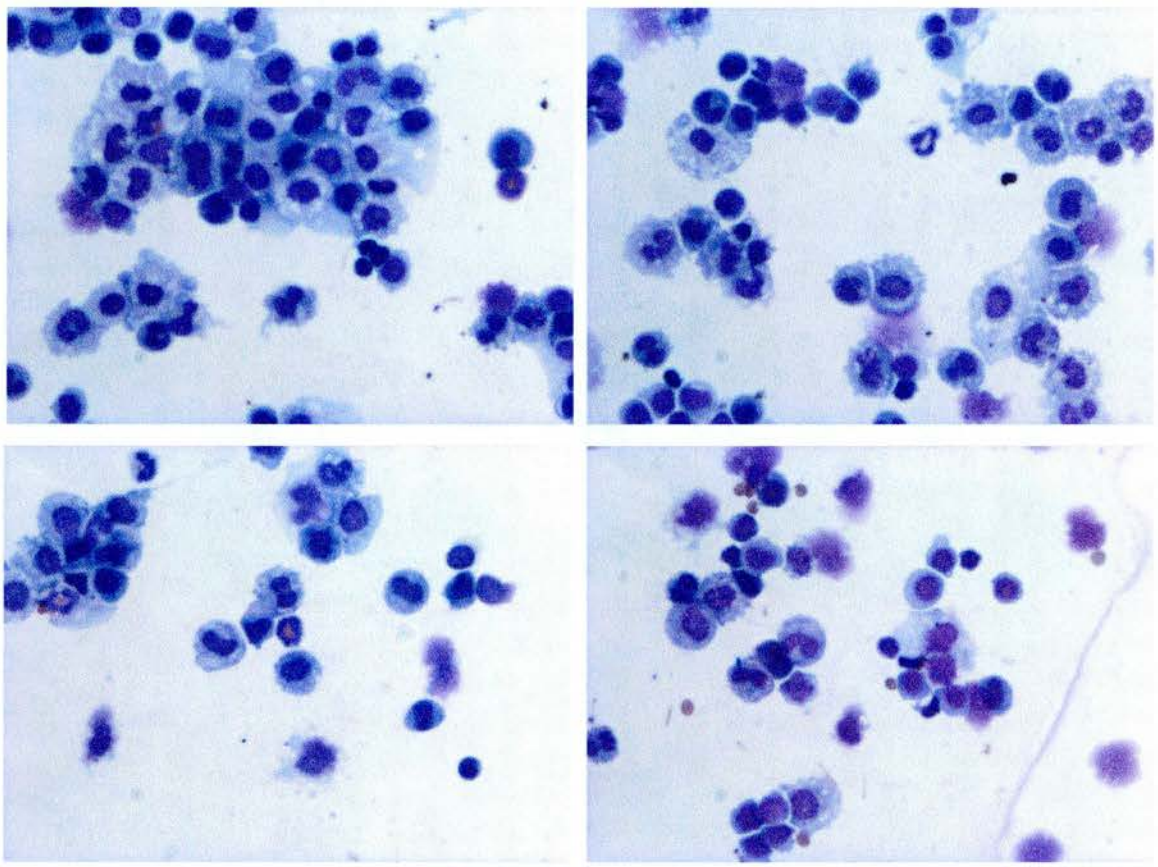


Figure 7-1: FACS analysis of Control lavages. Peritoneal cells were lavaged from control WT and 11β-HSD1-deficient mice. $0.5X10^6$ cells were labelled with F4/80 Ab, GR-1 Ab or IgG2b PE isotype control Ab as described in methods, and 10,000 events were collected for each of 4 mice of each genotype. A; unstained cells separate- on the basis of size- into 3 distinct populations, R7, R8 and R9. The ungated population is debris. B; histogram showing 4 isotype controls (black) overlayed on 4 F4/80 Ab stained samples (multicoloured) of each genotype for each gated population. C; histogram showing 4 isotype controls (black) overlayed on 4 GR-1 Ab stained samples (multicoloured) of each genotype for each gated population. GR-1 positivity is due to blood contamination of a single lavage during surgery.

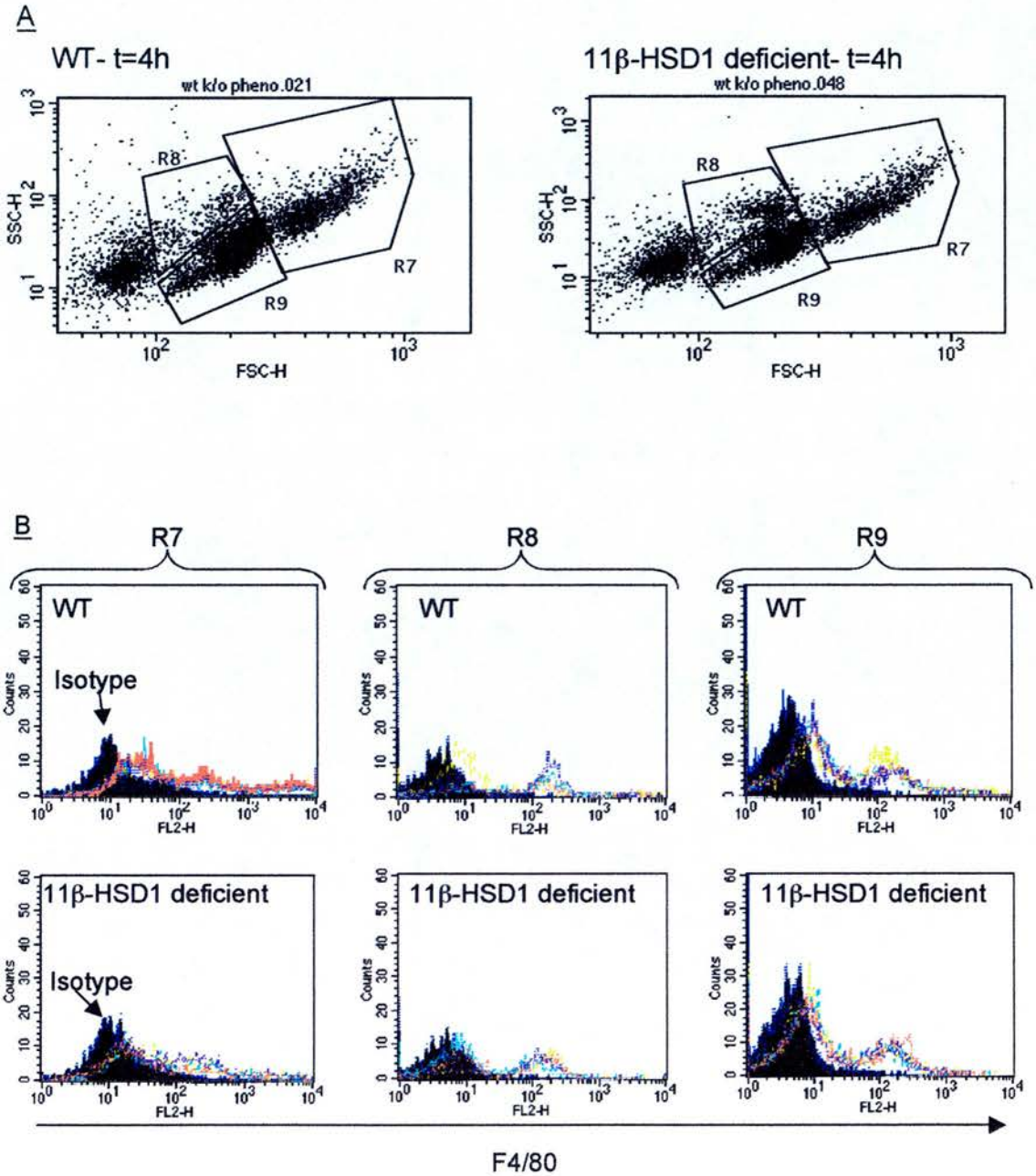
A- WT



B- 11β-HSD1-deficient



(Previous page)
Figure 7-2: Images of WT and 11β-HSD1-deficient mice. Control. Cytospins showing cell types present in the peritoneums of A, healthy WT and B, 11β-HSD1-deficient C57 BL/6. Each panel is a representative photograph of each of 4 mice. Note that the low numbers of red blood cells present are from the result of surgery and not normally present in the peritoneum.


A
WT- t=4h
wt k/o pheno.021
R8
R7
R9
SSC-H
FSC-H
11β-HSD1 deficient- t=4h
wt k/o pheno.048
R8
R7
R9
SSC-H
FSC-H
B
R7
R8
R9
WT
Isotype
11β-HSD1 deficient
Isotype
Counts
FL2-H
F4/80

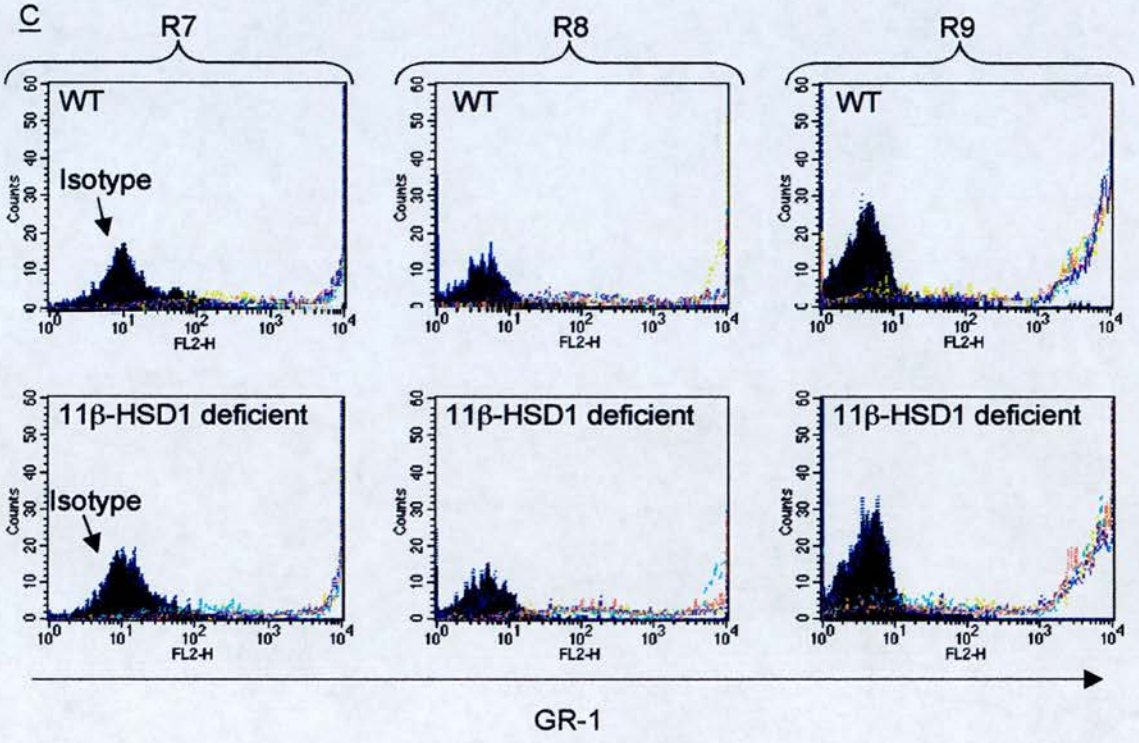
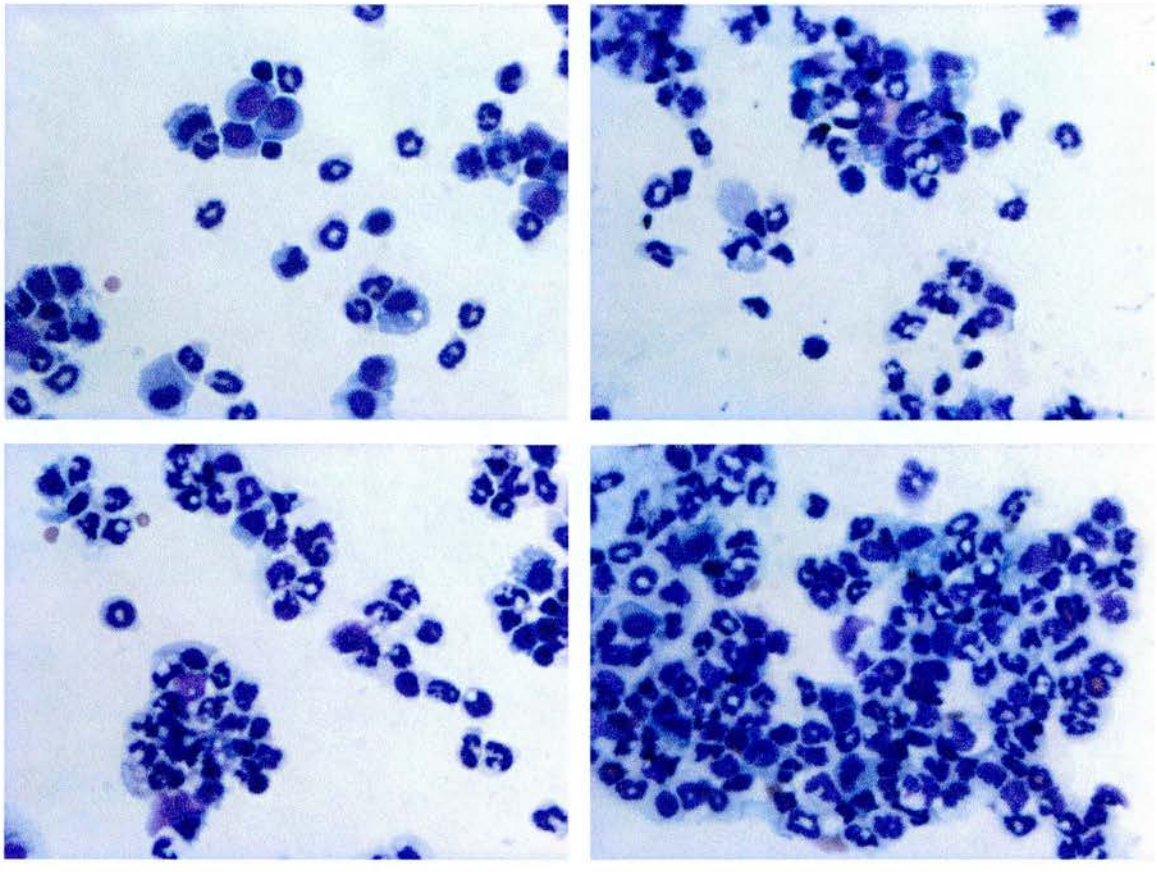
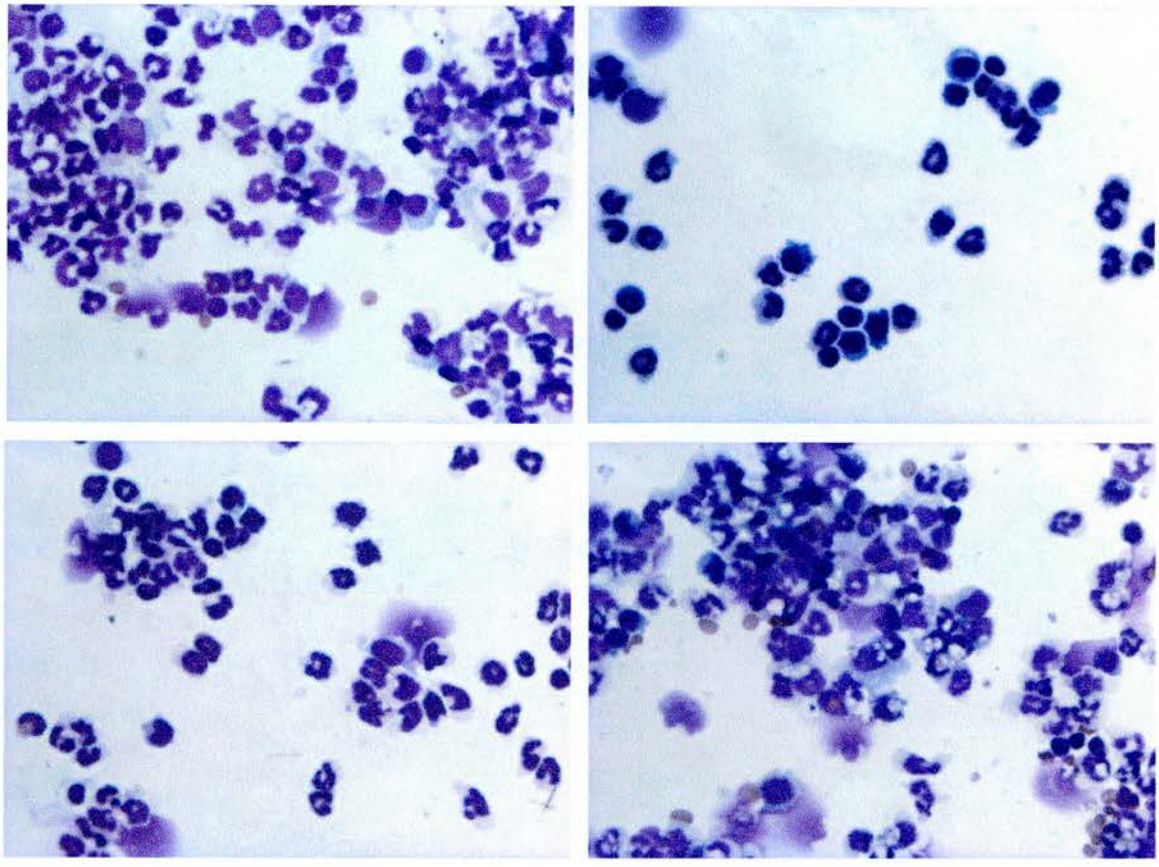


Figure 7-3: FACS analysis of lavages 4h after onset of peritonitis. Peritoneal cells were lavaged from WT and 11β-HSD1-deficient mice. $0.5X10^6$ cells were labelled with F4/80 Ab, GR-1 Ab or IgG2b PE isotype control Ab as described in methods, and 10,000 events were collected for each of 4 mice of each genotype. A; unstained cells separate- on the basis of size- into 3 distinct populations, R7, R8 and R9. The ungated population is debris. B; histogram showing 4 isotype controls (black) overlayed on 4 F4/80 Ab stained samples (multicoloured) of each genotype for each gated population. Note that the F4/80 positivity is between the 2nd and 3rd log order. C; histogram showing 4 isotype controls (black) overlayed on 4 F4/80 Ab stained samples (multicoloured) of each genotype for each gated population.

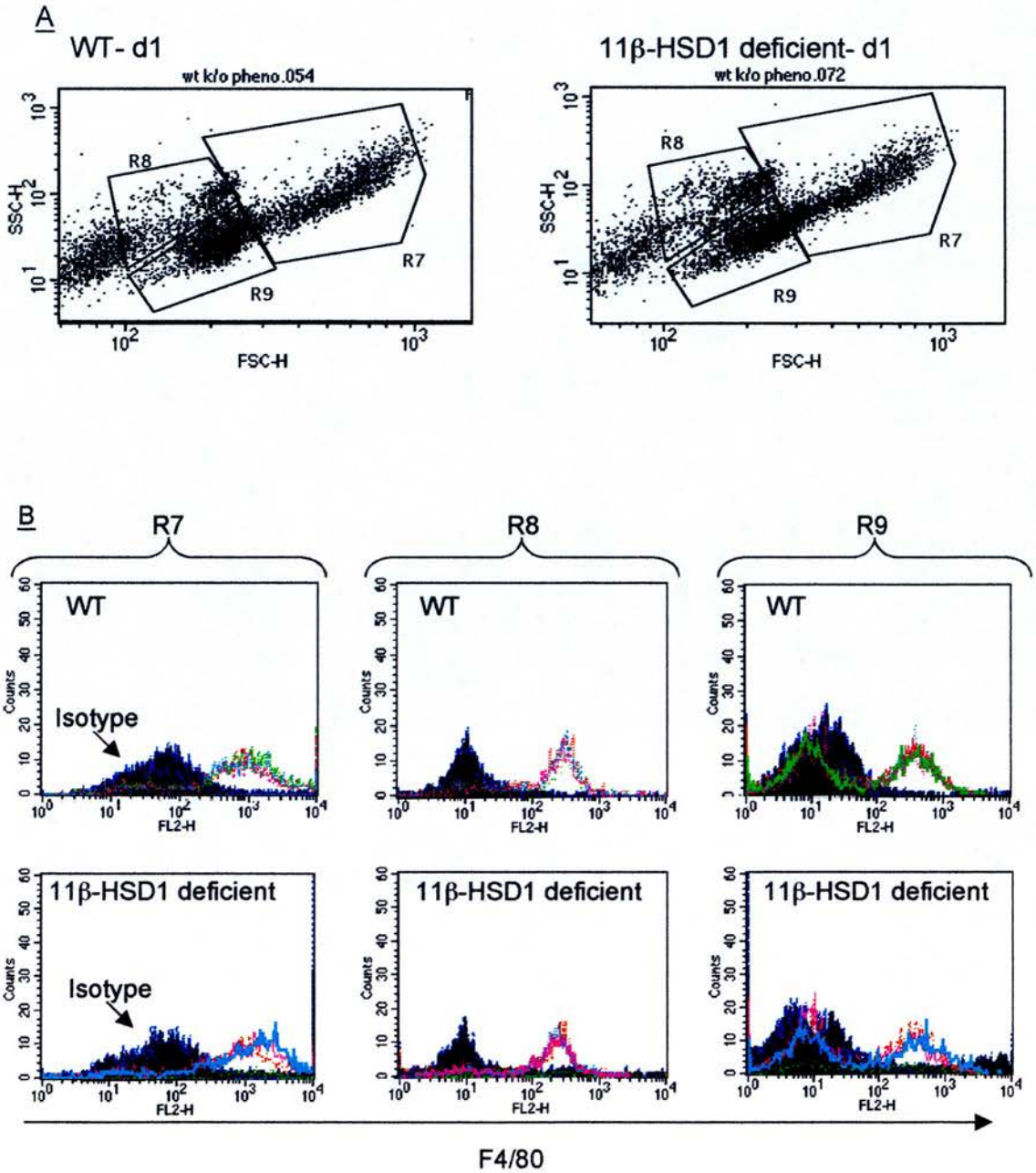
A- WT



B- 11β-HSD1-deficient



(Previous page)
Figure 7-4: Images of lavage cytospins 4h after onset of peritonitis. Cytospins showing cell types present in A, WT and B, 11β-HSD1-deficient peritoneums 4h after i.p injection of 3% thioglycollate. Each panel is a representative photograph of each of 4 mice.


A
WT- d1
wt k/o pheno.054
R8
R7
R9
SSC-H
FSC-H
11β-HSD1 deficient- d1
wt k/o pheno.072
R8
R7
R9
SSC-H
FSC-H
B
R7
R8
R9
WT
Isotype
11β-HSD1 deficient
Isotype
Counts
FL2-H
F4/80

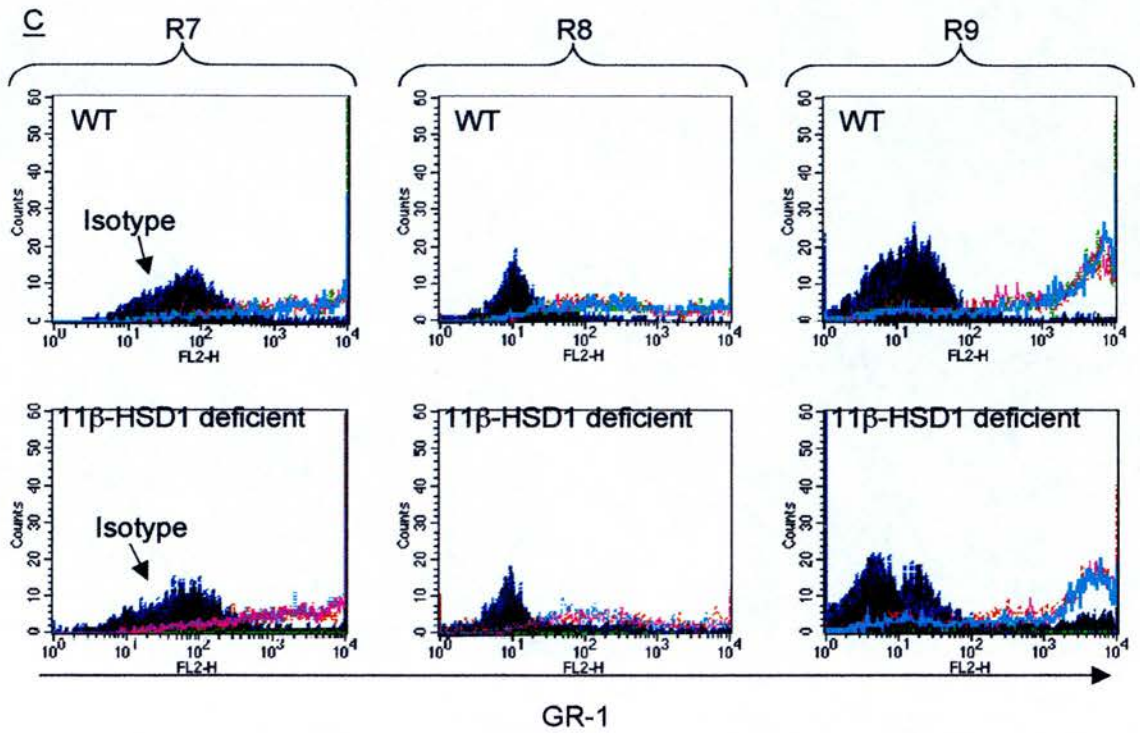
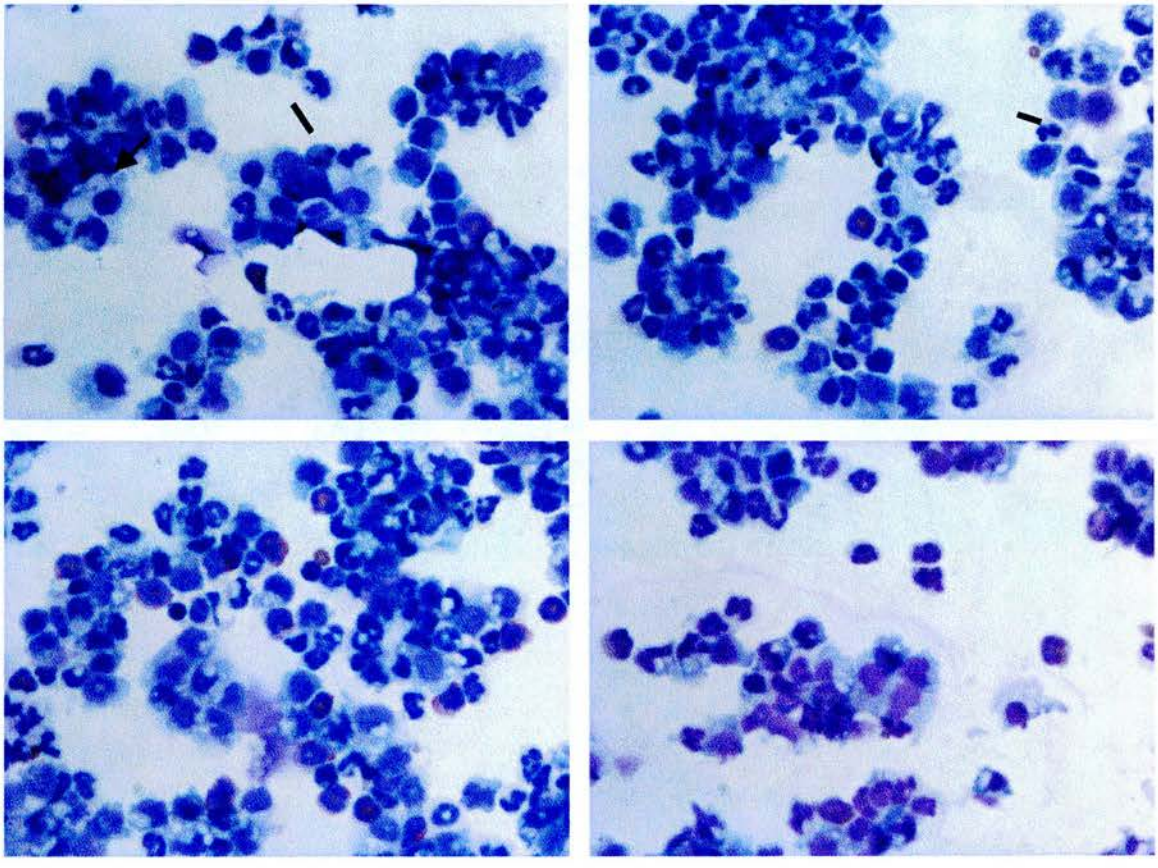
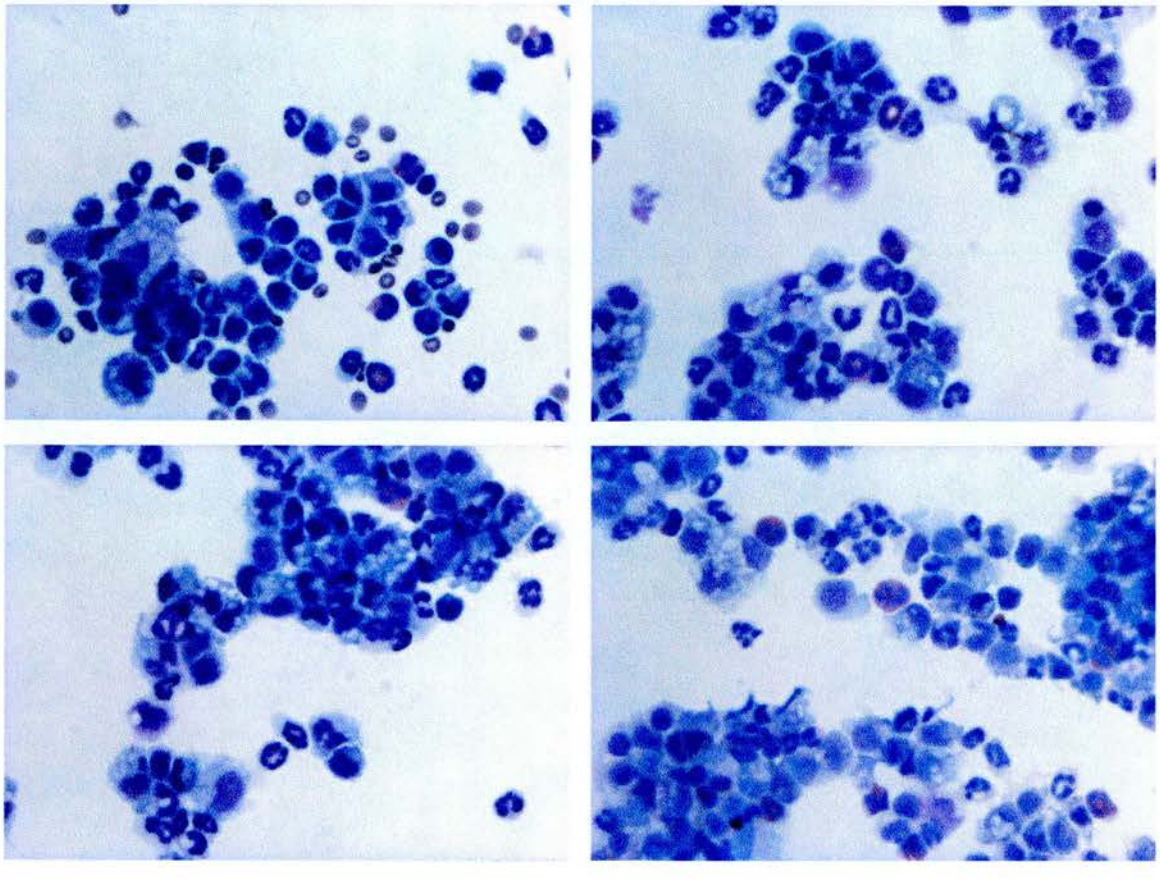


Figure 7-5: FACS analysis of lavages 1d after onset of peritonitis. Peritoneal cells were lavaged from WT and 11β-HSD1-deficient mice. $0.5X10^6$ cells were labelled with F4/80 Ab, GR-1 Ab or IgG2b PE isotype control Ab as described in methods, and 10,000 events were collected for each of 4 mice of each genotype. A; unstained cells separate- on the basis of size- into 3 distinct populations, R7, R8 and R9. The ungated population is debris. B; histogram showing 4 isotype controls (black) overlayed on 4 F4/80 Ab stained samples (multicoloured) of each genotype for each gated population. C; histogram showing 4 isotype controls (black) overlayed on 4 GR-1 Ab stained samples (multicoloured) of each genotype for each gated population.

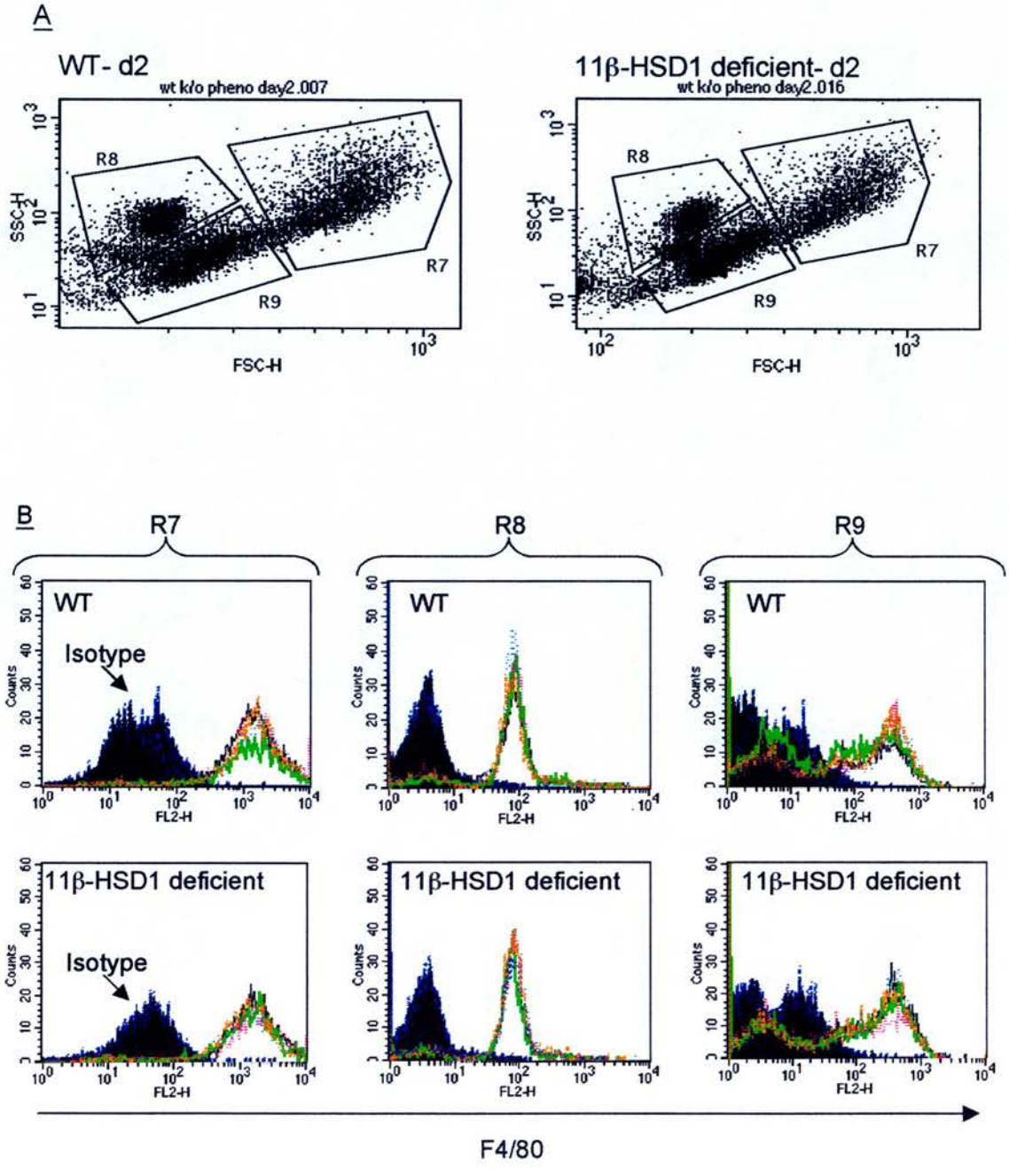
A- WT



B- 11β-HSD1-deficient



(Previous page)
Figure 7-6: Images of lavage cytospins 1d after onset of peritonitis. Cytospins showing cell types present in A, WT and B, 11β-HSD1-deficient peritoneums 1d after i.p injection of 3% thioglycollate. Each panel is a representative photograph of each of 4 mice. Note the increased numbers of free apoptotic cells (l) and Mϕs that contain apoptotic bodies (→) in the WT populations.


A
WT- d2
wt k/o pheno day2.007
R8
R7
R9
SSC-H
FSC-H
11β-HSD1 deficient- d2
wt k/o pheno day2.016
R8
R7
R9
SSC-H
FSC-H
B
R7
R8
R9
WT
Isotype
11β-HSD1 deficient
Isotype
Counts
FL2-H
F4/80

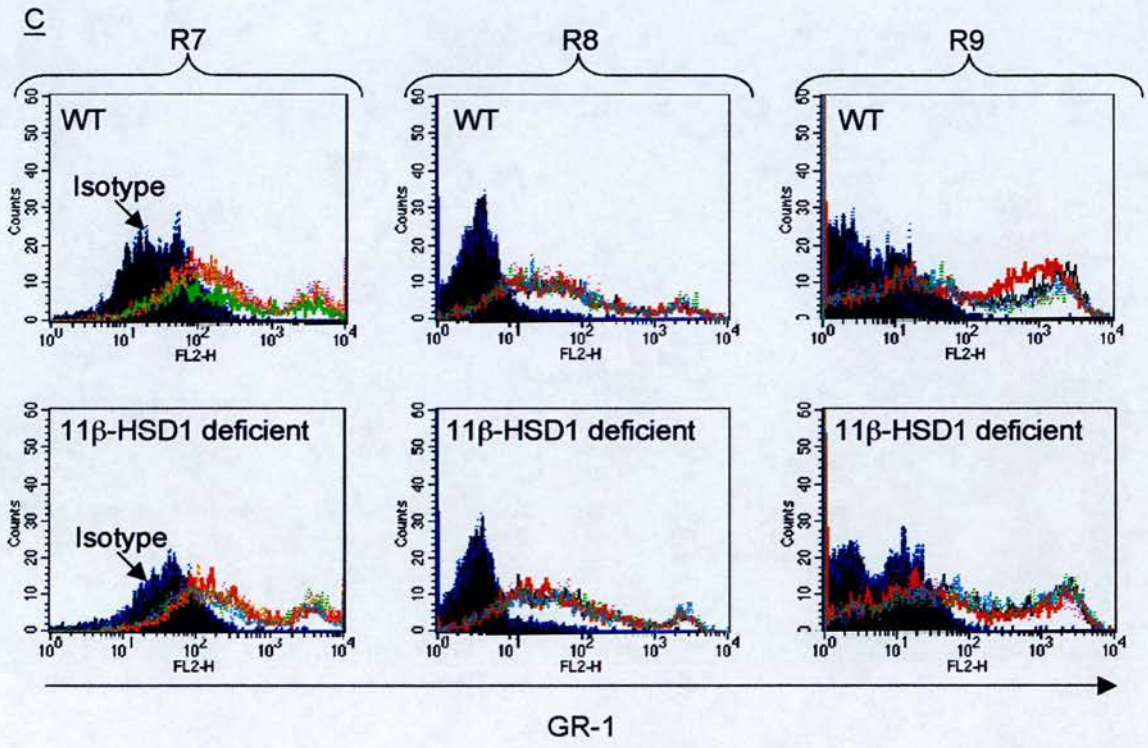
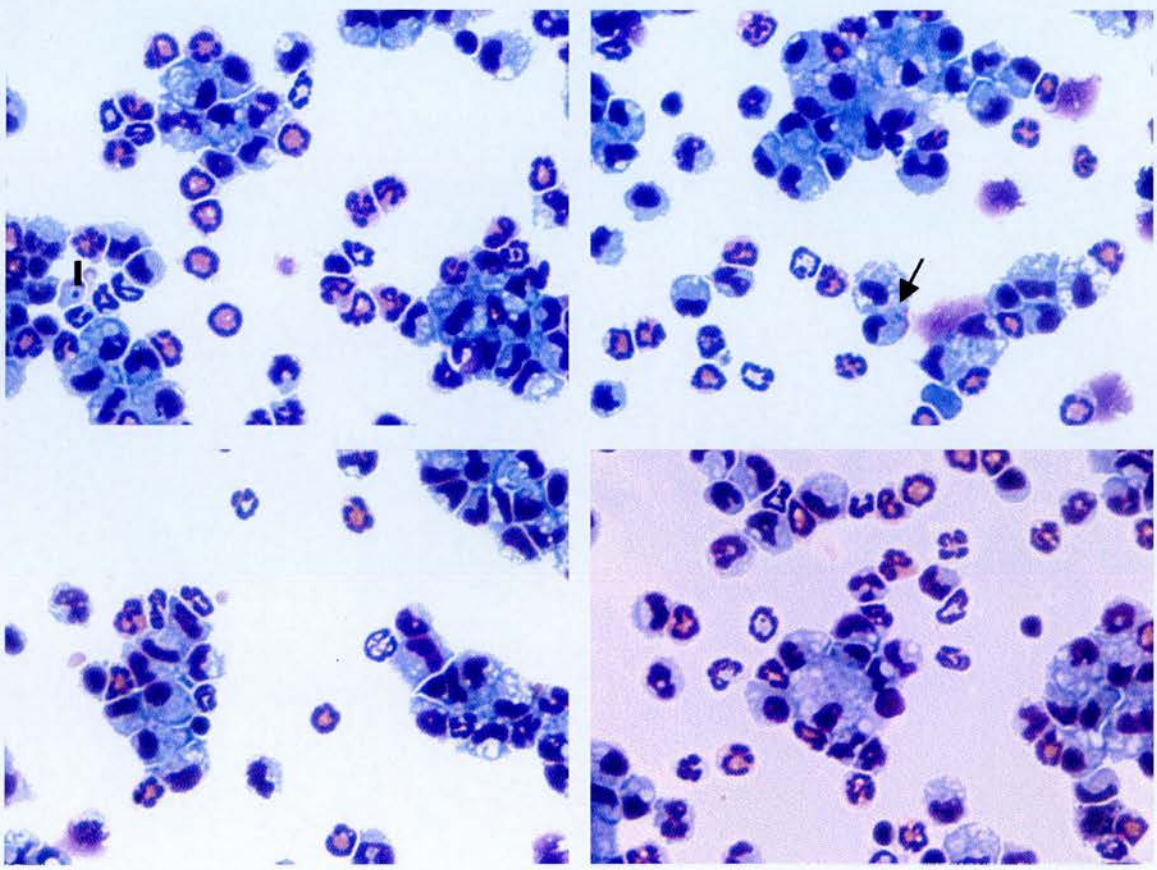
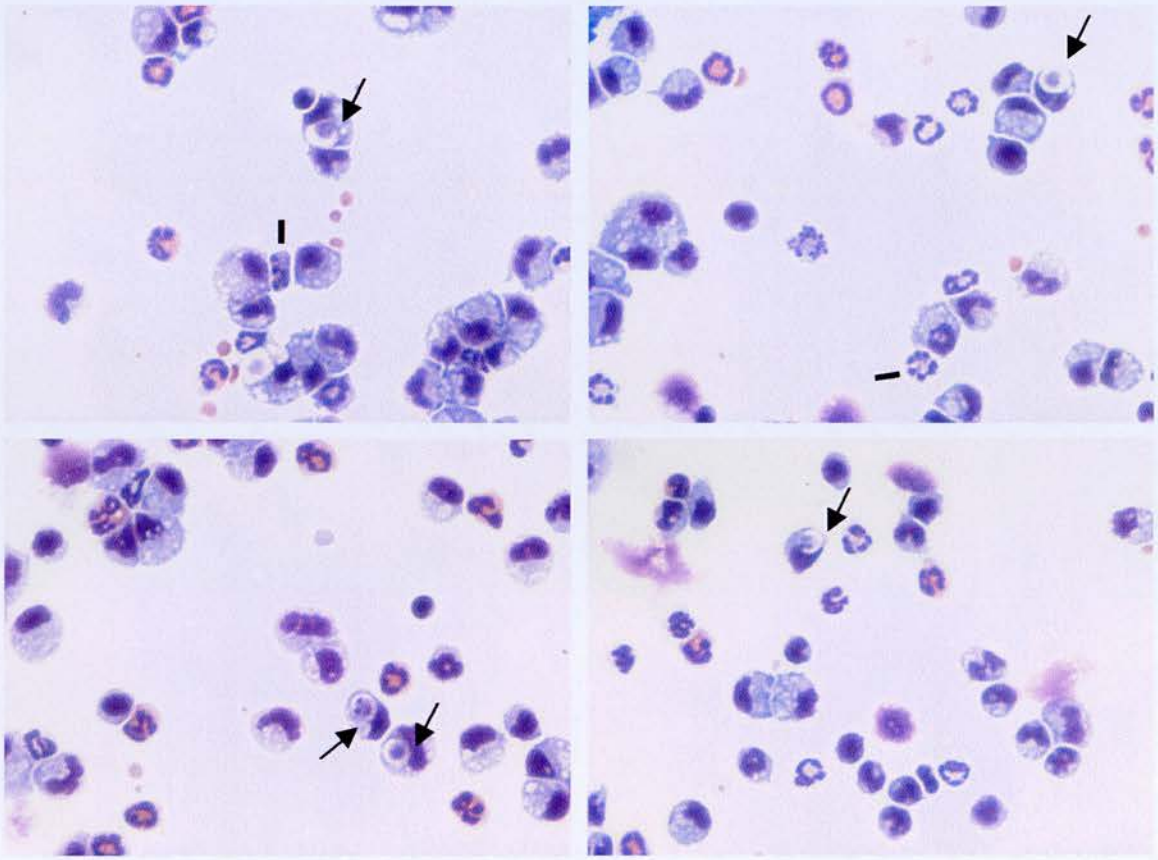


Figure 7-7: FACS analysis of lavages 2d after onset of peritonitis. Peritoneal cells were lavaged from WT and 11β-HSD1-deficient mice. $0.5X10^6$ cells were labelled with F4/80 Ab, GR-1 Ab or IgG2b PE isotype control Ab as described in methods, and 10,000 events were collected for each of 4 mice of each genotype. A; unstained cells separate- on the basis of size- into 3 distinct populations, R7, R8 and R9. The ungated population is debris. B; histogram showing 4 isotype controls (black) overlayed on 4 F4/80 Ab stained samples (multicoloured) of each genotype for each gated population. C; histogram showing 4 isotype controls (black) overlayed on 4 GR-1 Ab stained samples (multicoloured) of each genotype for each gated population.

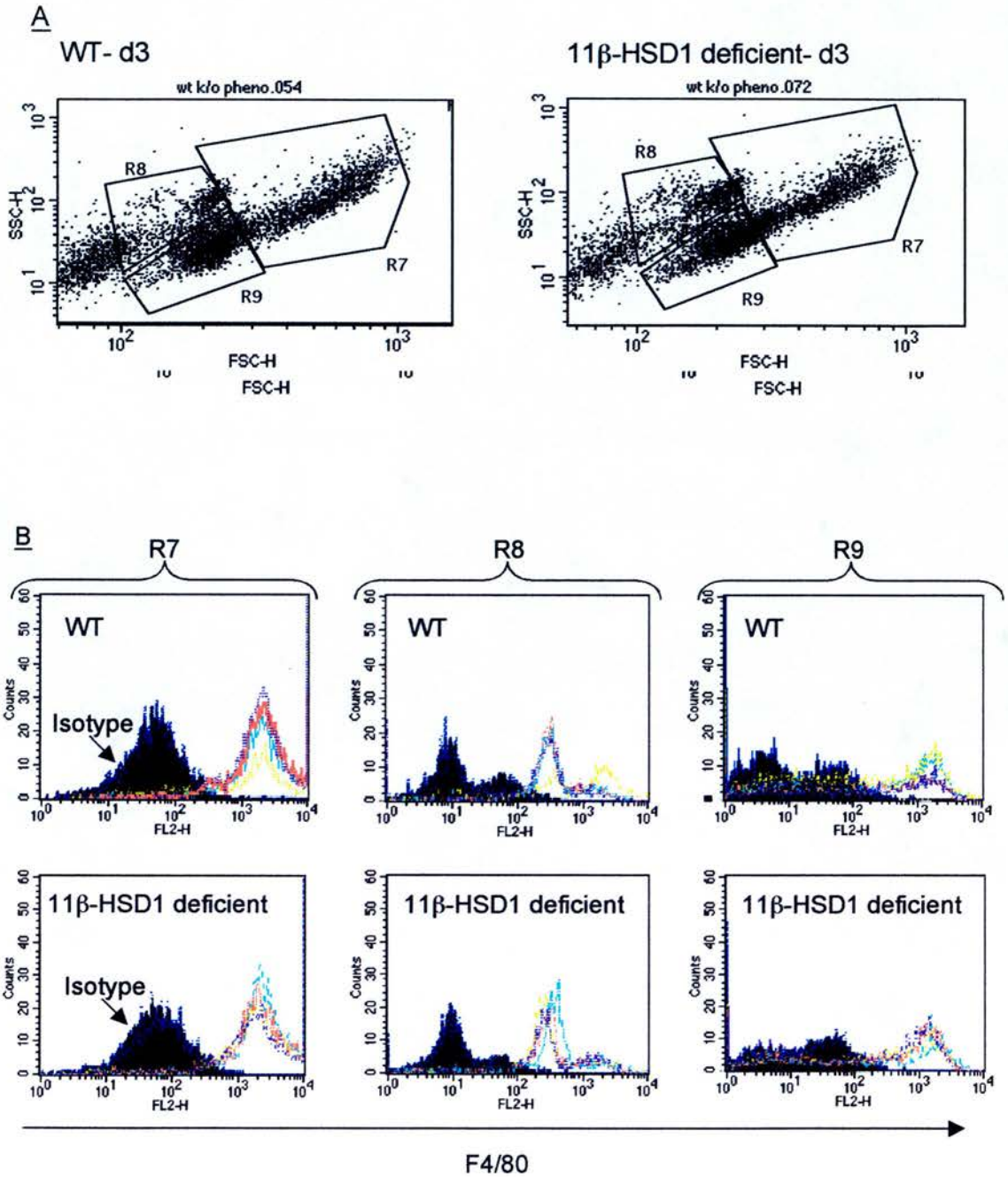
A- WT



B- 11β-HSD1-deficient



(Previous page)
Figure 7-8: Images of lavage cytospins 2d after onset of peritonitis. Cytospins showing cell types present in A, WT and B, 11β-HSD1-deficient peritoneums 2d after i.p injection of 3% thioglycollate. Each panel is a representative photograph of each of 4 mice. Note the increased numbers of free apoptotic cells (l) and Mϕs that contain apoptotic bodies (→) in the 11β-HSD1-deficient populations.


A
WT- d3
wt k/o pheno.054
R8
R7
R9
SSC-H
FSC-H
11β-HSD1 deficient- d3
wt k/o pheno.072
R8
R7
R9
SSC-H
FSC-H
B
R7
R8
R9
WT
Isotype
Counts
FL2-H
11β-HSD1 deficient
Isotype
Counts
FL2-H
F4/80

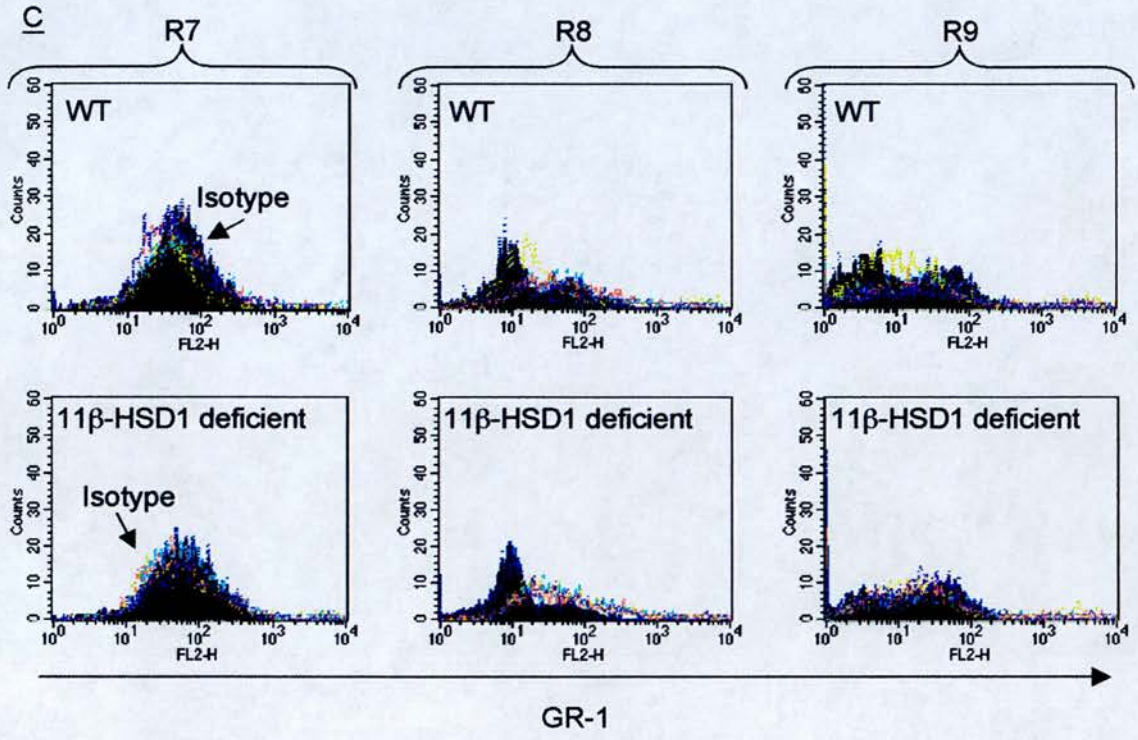
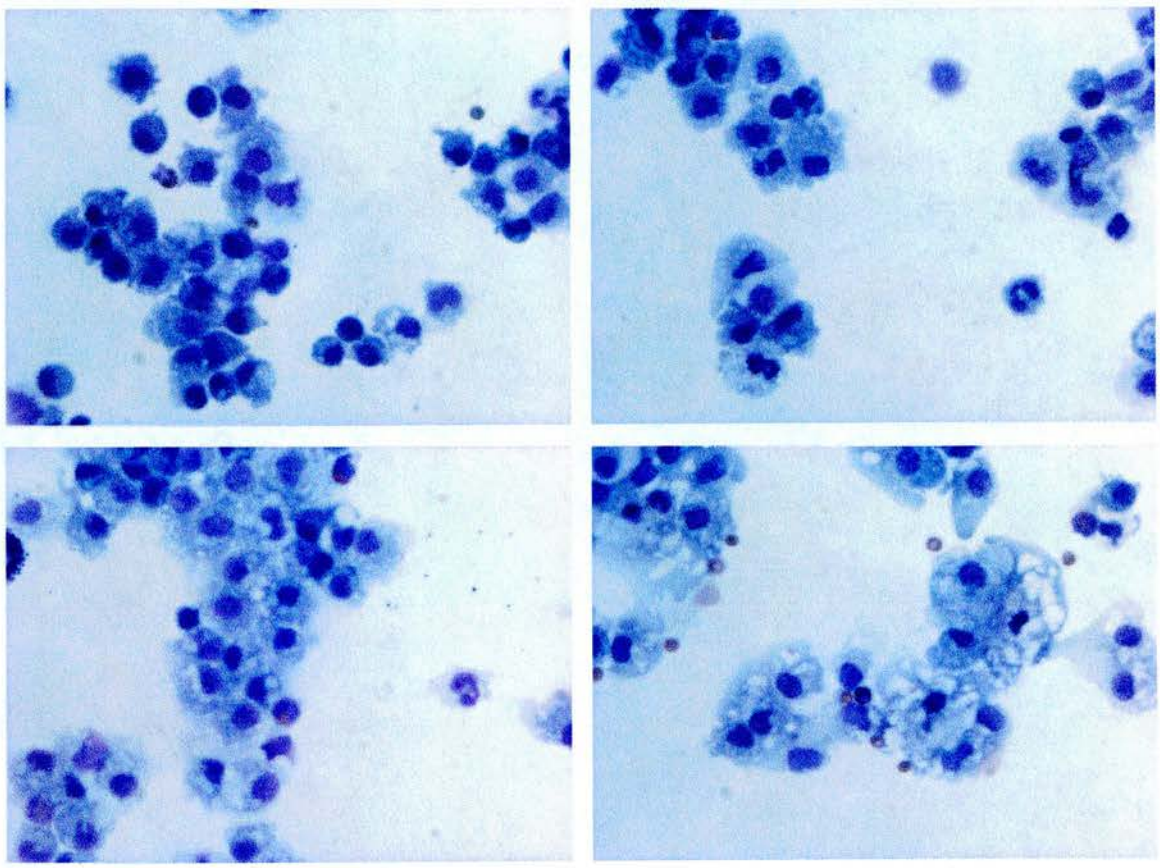
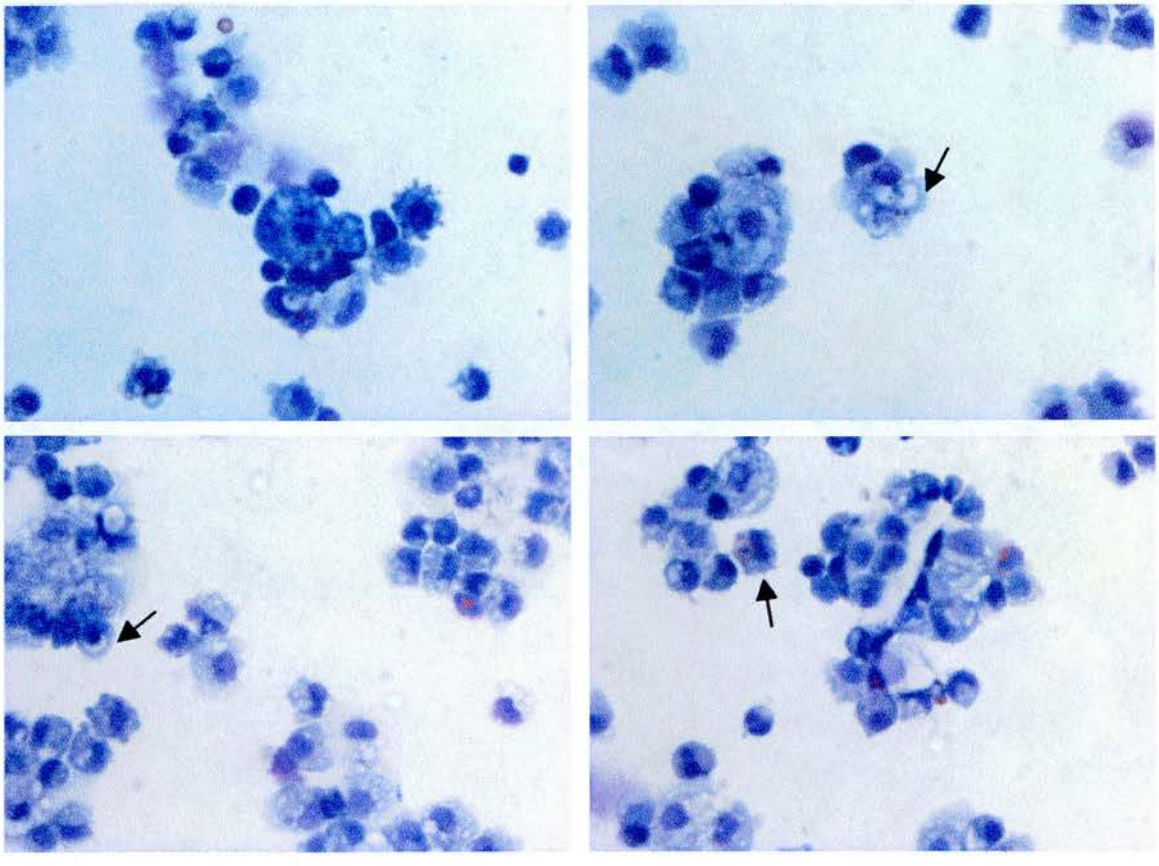


Figure 7-9: FACS analysis of lavages 3d after onset of peritonitis. Peritoneal cells were lavaged from WT and 11β-HSD1-deficient mice. $0.5X10^6$ cells were labelled with F4/80 Ab, GR-1 Ab or IgG2b PE isotype control Ab as described in methods, and 10,000 events were collected for each of 4 mice of each genotype. A; unstained cells separate- on the basis of size- into 3 distinct populations, R7, R8 and R9. The ungated population is debris. B; histogram showing 4 isotype controls (black) overlayed on 4 F4/80 Ab stained samples (multicoloured) of each genotype for each gated population. C; histogram showing 4 isotype controls (black) overlayed on 4 GR-1 Ab stained samples (multicoloured) of each genotype for each gated population.

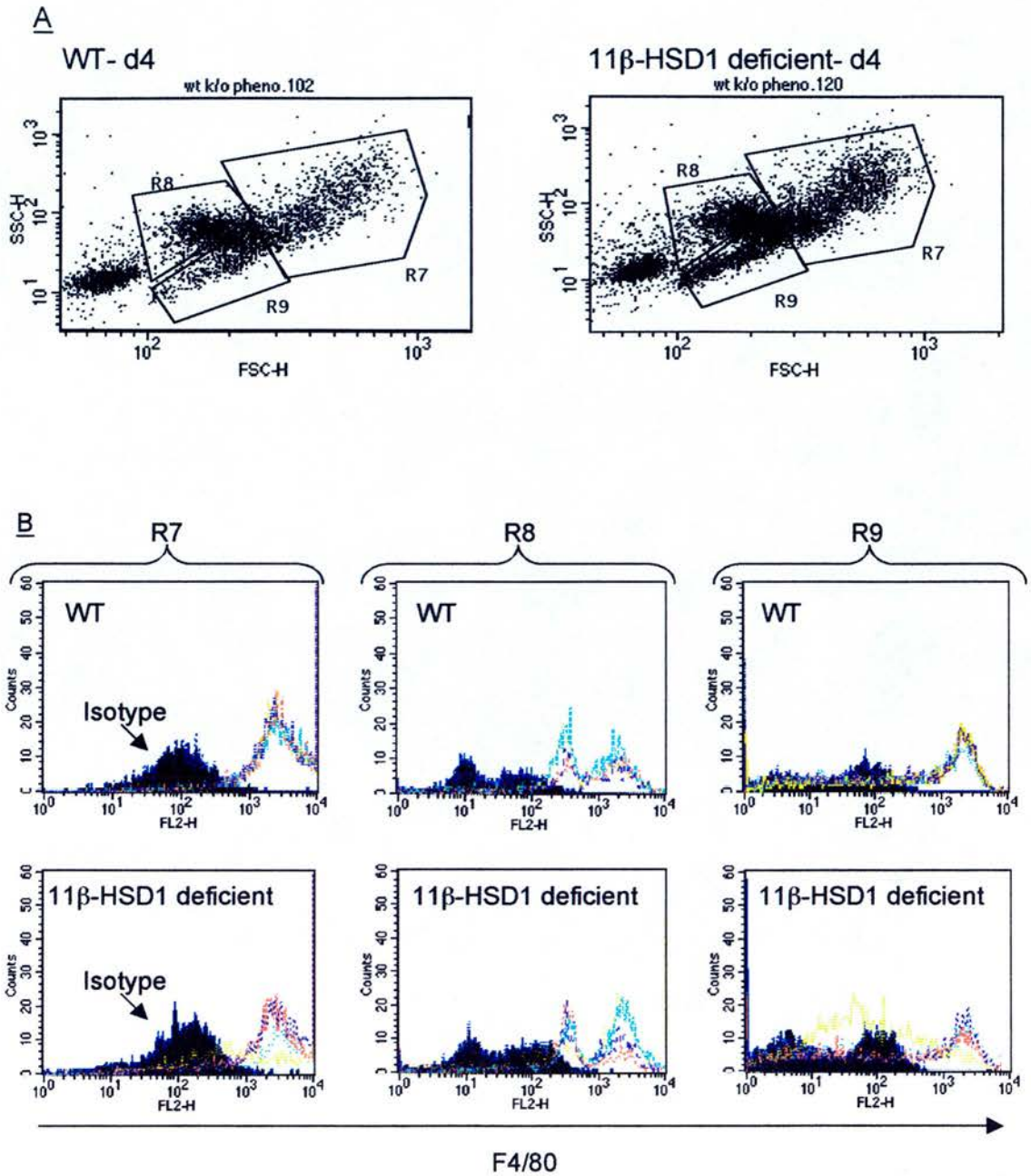
A- WT



B- 11β-HSD1-deficient



(Previous page)
Figure 7-10: Images of lavage cytospins 3d after onset of peritonitis. Cytospins showing cell types present in A, WT and B, 11β-HSD1-deficient peritoneums d3 after i.p injection of 3% thioglycollate. Each panel is a representative photograph of each of 4 mice. Note the increased numbers of Mϕs that contain apoptotic bodies (→) in the 11β-HSD1-deficient populations.


A
WT- d4
wt k/o pheno.102
R8
R7
R9
SSC-H
FSC-H
11β-HSD1 deficient- d4
wt k/o pheno.120
R8
R7
R9
SSC-H
FSC-H
B
R7
R8
R9
WT
Isotype
11β-HSD1 deficient
Isotype
Counts
FL2-H
F4/80

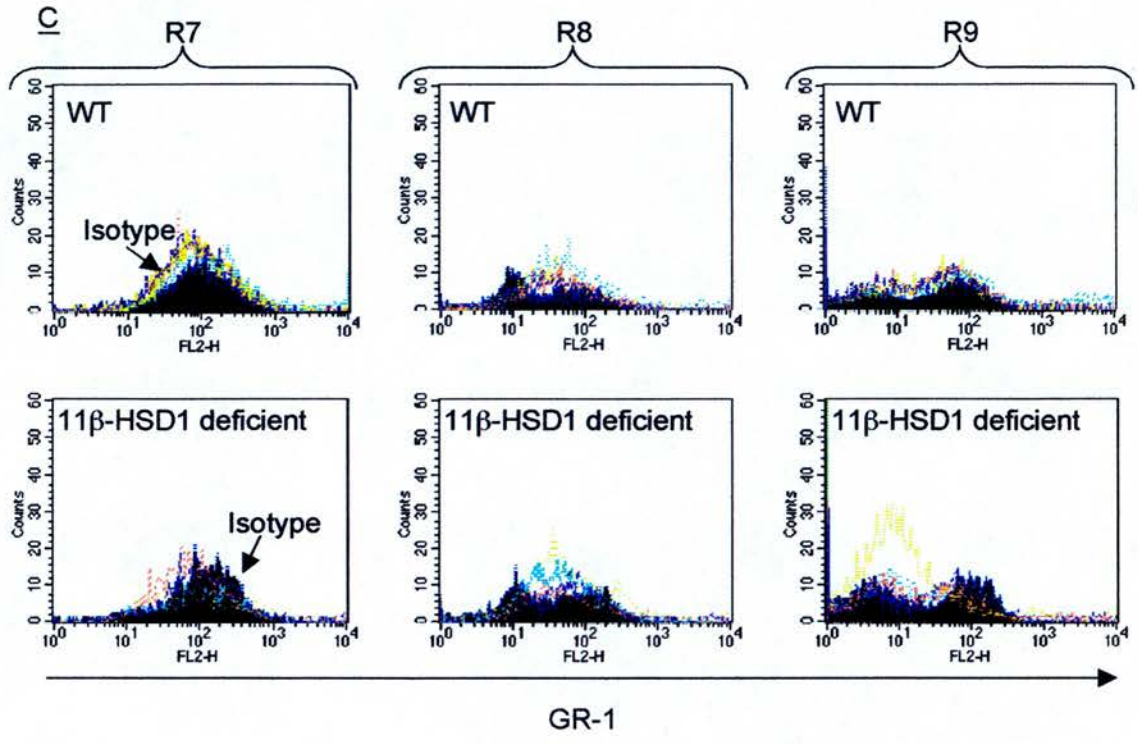
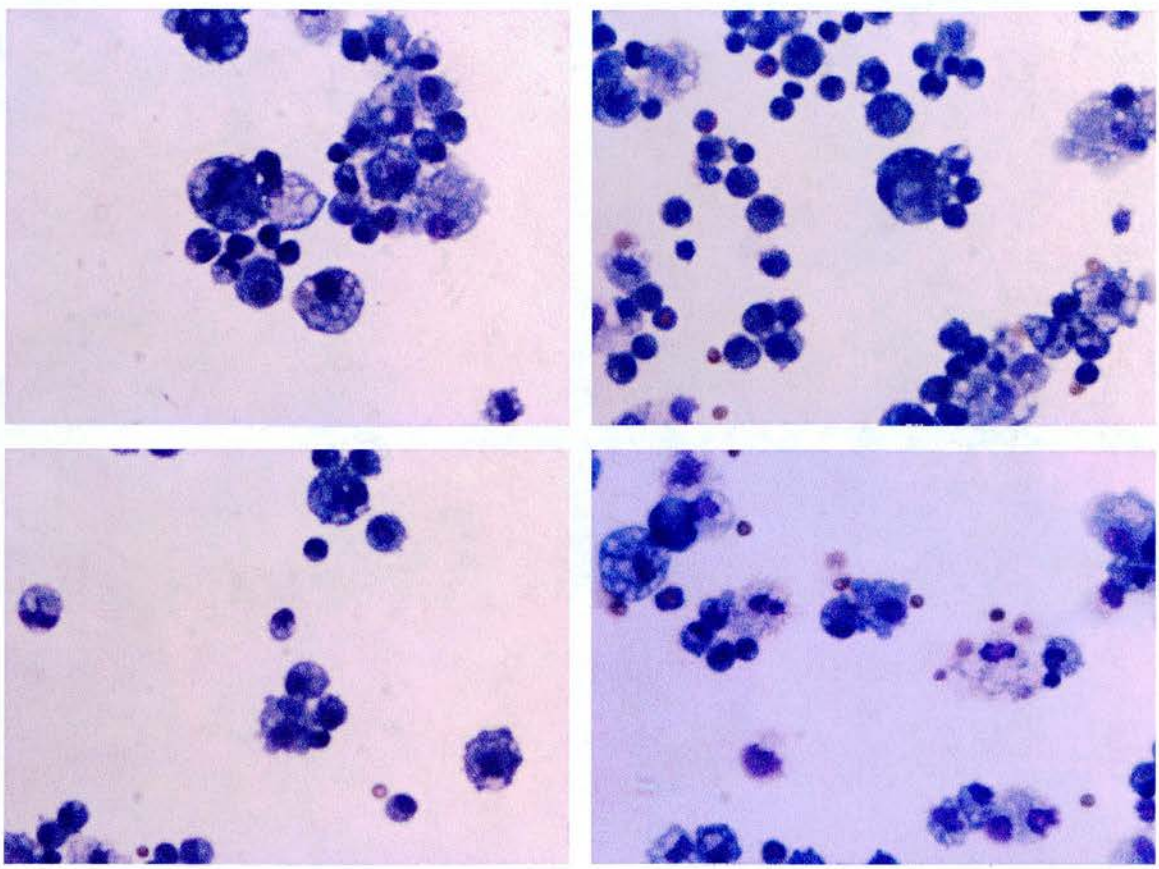
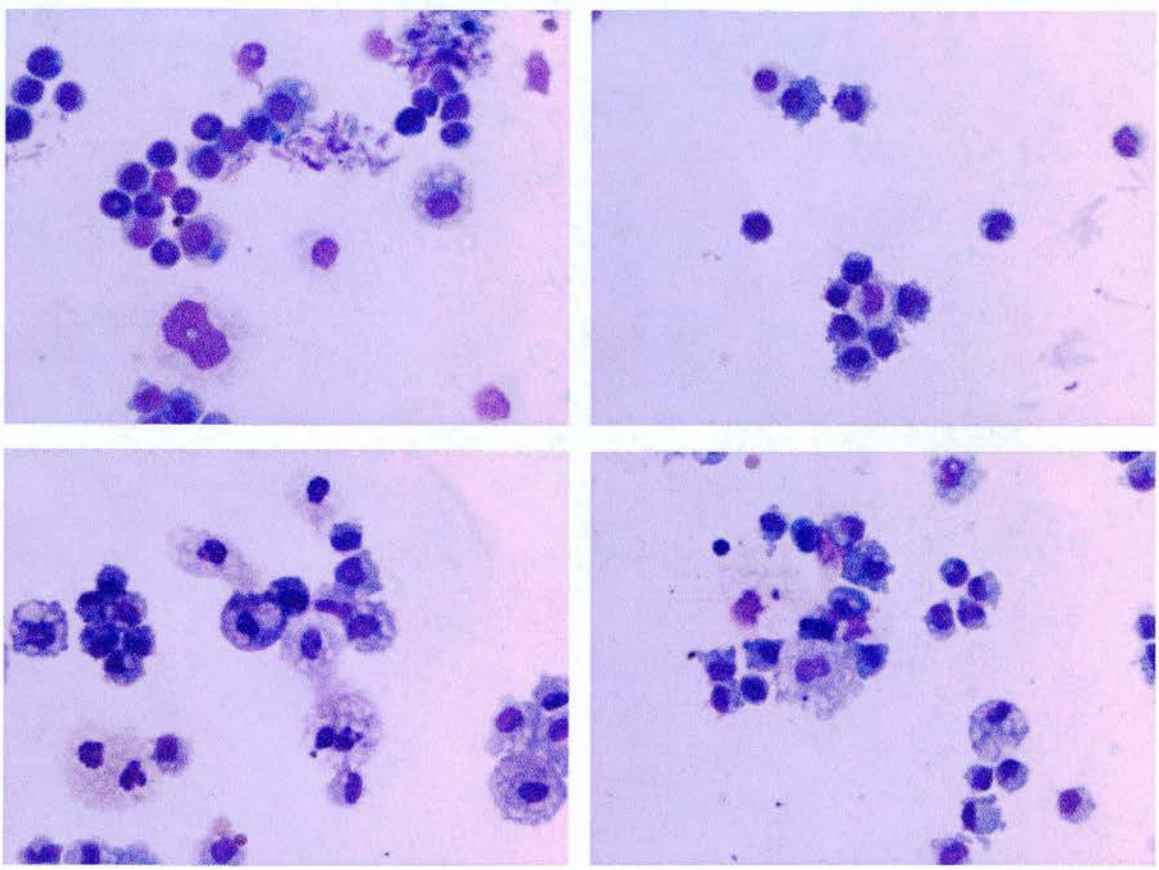


Figure 7-11: FACS analysis of lavages 4d after onset of peritonitis. Peritoneal cells were lavaged from WT and 11β-HSD1-deficient mice. $0.5X10^6$ cells were labelled with F4/80 Ab, GR-1 Ab or IgG2b PE isotype control Ab as described in methods, and 10,000 events were collected for each of 4 mice of each genotype. A; unstained cells separate- on the basis of size- into 3 distinct populations, R7, R8 and R9. The ungated population is debris. B; histogram showing 4 isotype controls (black) overlayed on 4 F4/80 Ab stained samples (multicoloured) of each genotype for each gated population. C; histogram showing 4 isotype controls (black) overlayed on 4 GR-1 Ab stained samples (multicoloured) of each genotype for each gated population.

A- WT



B- 11β-HSD1-deficient



(Previous page)
Figure 7-12: Images of lavage cytospins 4d after onset of peritonitis. Cytospins showing cell types present in A, WT and B, 11β-HSD1-deficient peritoneums 4d after i.p injection of 3% thioglycollate. Each panel is a representative photograph of each of 4 mice.